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Gene Silencing in Mammalian Cells

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Thesis submitted to the Department of Biochemistry in partial fulfillment of the requirements for the degree of Master of Science

Department of Biochemistry
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Canadä

Gametic Imprinting is the unequal expression of genes from the male and female alleles that results in parental dependant traits. We report here of an unusual Pgk-1.2-lacZ transgenic line that is irreversibly inactivated when passaged through the female germline. Inactivation of the transgene is correlated with methylation. HpaII sites within the lacZ region of the inactivated transgene are completely methylated. and treatment of mouse embryonic fibroblasts carrying inactivated transgenes with 5-aza-2-deoxycytidine results in reactivation.

Additionally. we used P19 embryonal carcinoma cells to examine transgene inactivation. as cultured cells are more amenable to manipulation. Once again. methylation was correlated with transgene inactivation. Cells uniformly expressing the Pgk-1.2-lacZ transgene are also methylated to a slightly lesser degree suggesting that within transgene arrays. only a subset of transgenes are actively transcribed.

This thesis is dedicated to the hundreds of mice whose lives were sacrificed for the purpose of my research.

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Thank You

I declare I am the sole author of this thesis. All research work was done by me with the exception of:

a. The work to produce Fig. 1 (Karen Jardine and Dr. Michael McBurney)
b. The matings of mice to determine reversibility of transgene imprint (Dr. Michael McBurney)
c. Fig 14 which was appended from Jeniffer Ingram
d. The production of new Pgk-1,2-lacZ founders (Dr. Michael McBurney)
e. Targeted Disruption of Sty ES cells and transgenic mice (Peter Duncan, Jennifer Ingram and Dr. John Bell)

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List of Abbreviations

CAT	Chloremphenicol Acetyl Transferase
CMV	Cytomegalo Virus
EC	Embryonal Carcinoma
EDTA	Ethylene-Diamine Tetra Acetic Acid
ES	Embryonal Stem
HMG	3-Hydroxy-3-Methylglutaryl
H-2K^b	Major Histocompatibility Complex
IGF2	Insulin Growth Factor 2
LCR	Locus Control Region
LIF	Leukemia Inhibitory Factor
MAR	Matrix Attachment Region
MECP	Methyl-CpG Binding Protein
MEF	Moues Embryonic Fibroblast
MEMα	Minimal Essential Medium
PBS	Phosphate Buffered Saline
Pc	Polycomb
PEV	Position Effect Variegation
PGK	Phosphoglycerate Kinase
SAR	Scaffold Attachment Region
TE	Tris-EDTA
XIC	X-Inactivation Center
XIST	X-Inactive Specific Transcription

Introduction

Epigenetic gene regulation is the transmission of a pattern of gene expression that results from mechanisms other than alteration of DNA sequence. It represents an important mechanism of gene regulation in many organisms, and recent advances suggest that similarities exists between seemingly distinct epigenetic processes in yeast, *Drosophila*, and mammals.[36]

In mammals, a developmental program is established during embryogenesis which ensures proper expression of tissue specific genes, parental specific expression of imprinted genes, and inactivation of one copy of the X chromosome in females. Disregulation of epigenetic gene inactivation mechanisms often results in disease. Silencing of tumour suppressors p53 and Rb are correlated with oncogenesis, and failure to inactivate imprinted genes are manifested as congenital diseases such as Prader Willi syndrome. It is thus important and necessary to understand epigenetic gene regulation as it pertains to both natural phenomena and diseased states.

X-Chromosome Inactivation

Human somatic cells contain 23 pairs of chromosomes, one pair being the sex chromosomes. Males have one X and one Y chromosome while females have two X chromosomes. Dosage compensation is a mechanism to alleviate potential phenotypic consequences that may occur as a result of a double dosage of an extra chromosome. Mammals achieve dosage compensation in female cells by the process of X chromosome inactivation or lyonization. One of two copies of the X chromosome in female cells becomes transcriptionally inactivated. Epigenetic modification to the inactive X chromosome is a

random process which is stable and clonally heritable. The inactive X chromosome is condensed into heterochromatin and is known as the Barr body, residing near the nuclear periphery and replicating late during the S phase.[110] It is also correlated with increased methylation [108] and under acetylation of Histone H4 [113].

Silencing of the X chromosome is thought to initiate at the X Inactivation Center (XIC). The XIC is an X-linked locus within band Xq13.2 and is thought to be localized to a 450kb region.[34] Derivatives of the X chromosome lacking XIC are unable to inactivate suggesting a essential requirement for the XIC.[104] X-inactive Specific Transcription or XIST is a candidate gene residing within the XIC that is has been implicated in the regulation of lyonization.[7] XIST is processed into a 17kb piece of untranslated RNA that is expressed from the inactive X chromosome. Female mice lacking XIST on the maternally derived X chromosome are phenotypically normal but express the paternal X chromosome in all cells. Paternally derived mutated XIST in females is lethal. Expression of both X chromosomes was present in extra-embryonic tissue and not in the embryo proper. suggesting an intolerance of double dosage towards embryo maintenance. This is supported by XO paternally inherited XIST mutants which are normal. Thus XIST seems to be required for proper inactivation of the X chromosome in females but is dispensable during spermatogenesis.[62] Unfortunately. the molecular mechanism of X inactivation remains elusive as yet.

Genomic Imprinting

Parental genetic information was thought to be transmitted and expressed equally. However, embryonic and genetic studies have shown that there are exceptions.

Parthenogenetic or gynogenetic and androgenetic embryos fail to develop normally suggesting unequal parental gametic contributions. Gametic imprinting refers to the unequal expression from male and female alleles that result in parental dependent traits. To date, 17 genes are known to be imprinted.[43]

Insulin growth factor 2 (Igf2) gene was the first imprinted gene to be characterized. Paternally transmitted mutant Igf2 in mice resulted in growth retarded offspring whereas maternal transmission of the mutant allele produced normal offspring. Therefore Igf2 is imprinted such that it is expressed only when paternally derived.[19] Interestingly, a second imprinted gene, H19, was found within close proximity to the Igf2 locus. H19 is exclusively maternally expressed.[114]

How does a cell distinguish which allele is to be expressed when there is no sequence difference between maternal and paternal alleles? Methylation of DNA has been proposed to be the imprinting mechanism as it has been correlated with gene inactivation.[36] Differences in methylation have only been identified within promoter elements of the paternal H19 gene.[28] There appears to be no discernible methylation difference between Igf2 parental alleles, yet, Igf2 is expressed exclusively from the paternal allele. Thus a simple correlation between methylation and expression appears to be absent at the Igf2 and H19 loci. A proposed model suggests that H19 and Igf2 compete for a common enhancer. There could be a preference for the paternal Igf2 gene to be active since promoter elements may be unavailable on the H19 gene due to methylation. When both promoters are unmethylated, there is a preference of the enhancer for the H19 promoter, which then results in exclusive expression of maternal H19 and repression of Igf2.[107] H19 mutant mice express maternal Igf2 and Ins2, another imprinted gene within close proximity that is

normally expressed paternally, when mutant H19 is maternally inherited. Normal expression remains upon paternal inheritance.[52] H19 appears then to be present to properly imprint nearby genes. An interesting point is that H19 and XIST genes produce no gene product. Expression of these RNAs results in the silencing of neighboring genes, the inactive X chromosome with XIST and maternal Igf2 with H19.

DNA Methylation

Methylation is the covalent modification of DNA at the C-5 position of cytosine resulting in 5-methylcytosine, and is catalyzed by DNA methyltransferase. It is the only known modification of DNA in vertebrates and has been correlated with gene inactivation.[108] Several lines of evidence support this notion.

Established during gametogenesis and early embryogenesis, methylation has been suggested to play a role in development.[59,79,109] Gene regulation during development could be controlled by processes of demethylation and de novo methylation allowing tissue and differentiation stage specific expression. Genes on the active and inactive X chromosome, as well as imprinted genes are differentially methylated.[67] Targeted mutation of DNA methyltransferase results in embryonic lethality suggesting that DNA methylation is essential to normal embryonic development but dispensable in stem cells.[58]

Hypermethylation of tumour suppressor genes such as p53, Rb, and p16CDKN2, and hypomethylation of proto-oncogenes are associated with tumourigenesis.[95,98] Congenital diseases such as Wilms' Tumour, Pradder-Willi, and Angleman and Beckwith-Wiedemann syndrome are associated with failure to inactivate imprinted genes.[83]

Methylation within promoter regions as well as transcribed regions of genes inhibit transcription in stable and transient transfections, in transcription assays in vitro [49] and in transgenic mice [86]. The use of demethylating reagents such as 5-Azacytidine alleviates gene and transgene inactivation supporting the notion that methylation is responsible for inactivation.[37]

Methylation is thought to induce gene inactivation through mechanisms which include chromatin remodeling, inhibition of transcription factor binding, and interaction with proteins which specifically bind methylated sequences [31]. These processes appear to be modulated by both methylation density and promoter strength [6].

Formation of active chromatin has been shown to be inhibited by DNA methylation. In fact experiments suggest that DNA methylation does not necessarily have to occur within the promoters of genes but may be present in the actual body of the gene or vector sequences to exert gene repression.[47-49] Endonuclease insensitivity and formation of higher order nucleosomal structures are associated with transfection of methylated constructs but not with unmethylated constructs.[47,49] Time dependent repression of transcription from methylated templates [47] and time dependent gene reactivation by 5-Azacytidine [39] are consistent with chromatin remodeling. Specific methylated sequences have been identified, which have been shown alter nucleosome positioning in vitro.[17] Furthermore evidence also indicates a preference for histone H1 by methylated DNA [64] irrespective of sequence [44,54]. Histone H1 is implicated in the formation of higher order repressive chromatin structures.[9,105]

Nucleosomes are the basic fundamental units of DNA packaging. A nucleosome unit is 146bp of DNA wrapped around a histone octamer (doublets of histones H2A, H2B, H3

and H4) with linker DNA of variable lengths between each nucleosome. The accessibility of promoter elements to transcription factors can be controlled by the presence of nucleosomes. Hence, the positioning of nucleosomes mediated by methylation [17,26,30] offers an attractive explanation as to how methylation exerts its effects on gene regulation. Reactivation of a chicken embryonic globin gene is dependent on treatment with 5-Azacytidine, which is amplified with subsequent sodium butyrate treatment. Sodium butyrate is an inhibitor of histone deacetylase and was not effective when administered alone.[29] These results are consistent with methylation induced nucleosome positioning which is initially alleviated by demethylation. Subsequent acetylation of residues on histone H3 and H4 increases accessibility of transcription factors to nucleosome bound DNA.[88]

Mechanistically, DNA methylation may also affect the binding of transcription factors, either directly or indirectly by interaction of methylated DNA with methylated DNA binding proteins. Firstly, recognition of transcription factor binding sites by regulatory proteins may be altered by methylation of cytosine residues that render them unrecognizable. Cyclic AMP responsive element binding protein [41] and two HeLa cell transcription factors [111] fail to recognize and bind to their respective DNA sequence elements when methylated. However, transcription factor Sp1 can bind and activate transcription from methylated and unmethylated templates equally well.[38] Secondly, nuclear proteins which preferentially bind methylated DNA may indirectly interfere with the transcriptional basal machinery. To date, only eight methylated DNA binding proteins or activities have been identified.; seven in vertebrates [82,99] and one in plants [25]. Methyl-CpG-Binding Protein 2 (MeCP2) is a 484 amino acid protein which binds to a symmetrical methyl-CpG pair in any sequence context, and is localized to pericentric chromatin.[56]

Targeted mutation of MeCP2 in mice were manifested in embryos with phenotypic abnormalities which failed to develop beyond mid-gestation.[103] Interestingly, similar to mutations in DNA methyltransferase, ES cells lacking MeCP2 are viable, suggesting that MeCPs are non-essential in stem cells.[77]

The density of methylation and promoter strength can influence the severity of repression. Transfected human and mouse globin genes which were differentially methylated were inhibited despite methylation density. Addition of an SV40 enhancer in cis relieved methylation mediated repression from low methylation density and not high density methylated templates.[6] This suggested that transcription of a gene is dependent on activating and repressive factors which compete for DNA binding. In vitro experiments using the same templates required methyl binding protein MeCP1 to obtain similar results.[6] Hence, as methylation density increases methylated DNA may serve as a sink for transcriptional repressive proteins which bind specifically to methylated DNA and displace activating proteins or prevent activating proteins from binding.

Gene Inactivation in Yeast

The first genetic experiments which implicated histones in gene regulation were performed in baker's yeast or Sacccharomyces cerevisiae. Histone H3 and H4 and regulatory proteins Sir2, Sir3, Sir4 and Rap1 have been implicated in the silencing of yeast mating type loci and genes integrated close to telomeres. The suggested model of inactivation at the telomeres is a polymerization of Sir3 and Sir4, recruited by Rap1, along nucleosomal arrays through interactions with the amino-terminal tails of histones H3 and H4.[35] Mutation of

any of these proteins abrogates silencing and/ or cell viability. These multiprotein complexes appear to be responsible for the maintenance of inactivation. [14,63]

The repressive effects of SIR proteins are dependent on concentration or gene dosage [61,63], suggesting that they are limiting with respect to the spreading of inactivation. Interestingly, increased amounts transcriptional activators has been shown to alleviate the repressive effects on telomere-proximal genes.[4] This may suggest a dynamic and competitive model that depends upon concentrations of repressive and activating factors, and that this competition may occur at the boundaries of the repressive complexes.

Gene Inactivation in *Drosophila*

Gene inactivation in *Drosophila* melanogaster is typically studied in terms of silencing related to relative position to heterochromatin (position effect variegation: PEV) [46], and local transcriptional silencing of genes which is not dependent on chromosomal location (ie. Polycomb Group mutimeric protein complex: Pc-G) [80].

PEV is the variable and reversible spreading of heterochromatin into a neighboring gene and is dependent on chromosomal position. At some point in development, the expression status of the euchromatic gene becomes heritable giving rise to mosaic expression pattern similar to X chromosome inheritance in mammals. Two models have been suggested for PEV. The first is similar to what occurs in yeast; packaging extending into the euchromatic gene from heterochromatin. The second, is the saturation of transcription activators by genes replicated early during S phase, thereby limiting those factors to genes replicated during Late S phase.[75]

The Polycomb (Pc) gene controls expression of homeotic genes. They exert their effects by silencing specific targets and locally inducing the formation of heterochromatin. Similar to experiments performed in yeast SIR3, Sir4, Rap1, and N-terminal histone acetyltransferases, mutations in Pc genes induce derepression of specific genes.[55]

Chromosomal induced silencing is relieved upon excision of a transgene from a PEV inducing chromosomal position in *Drosophila* somatic cells.[1] Transgenes are expressed transiently but subject to PEV once integrated into chromatin. These results are consistent with the spreading of inactivation and its maintenance in a chromatin environment

Although the mechanism of chromatin remodeling may be similar between yeast and *Drosophila* they do not involve homologous non-histone proteins. Similarity extends to the formation of multiprotein complexes which contain silencing proteins. These complexes are indirectly tethered to DNA through histone and non-histone proteins, and exclude transcription factors from essential promoter elements.

Gene Inactivation in Mammals

There are two natural phenomena of gene inactivation in mammals, X chromosome inactivation and genomic imprinting. Methylation is the likely candidate for these epigenetic phenomena. Transgenic mice and DNA recombination into cultured cells have been widely used to study these phenomena.

Cultured Cells

The advantage of using cultured cells is the ease at which they can be manipulated. Introduction of plasmid DNAs into cells has become a routine procedure. Unfortunately maintenance of stable and uniform expression appears to be a major caveat, especially in

undifferentiated cell types such as embryonal stem (ES) cells, and embryonal carcinoma (EC) cells.[68,94] Regardless of promoter, reporter genes, or selective agents, EC cells show a mosaic expression of transfected genes, which appears to be the result of both gene inactivation and gene loss.[94] Random integration or targeted homologous recombination into the active HPRT locus, in either orientation, by electroporation of a lacZ transgene into ES cells results in heterogeneous or mosaic expression.[96]

Introduction of transgenes driven by partially methylated metallotheinein 1 and HsvTK promoters, results in expression in F9 cells but not in mouse L cells. A methylated non-transcribed competitor co-transfected into L cells was able to alleviate that repression.[53] This suggests that L cells contain proteins that selectively bind methylated DNA, and that these factors are absent or low in abundance in undifferentiated cell types. The fact that ES cells are viable in the absence of MeCP2 [102], a methyl DNA binding protein, supports this notion.

Expression of transgenes within differentiated cell types appears to be homogeneous but their expression can be modulated by methylation. Transient transfections of methylated templates are initially expressed but lose expression in a time dependent fashion, consistent with the removal of transcriptional machinery from active templates.[47] Methylation of non- promoter elements such as vector sequences, or transcribed regions also results in repression [8,47,49] indicating the spreading of inactivation from a nucleation center, which parallel mechanisms of inactivation in *Drosophila* and yeast.

The nature of transgene integration has been suggested to signal inactivation of foreign DNAs. Transfection or pronuclei microinjection of plasmid DNAs into cells often results in multicopy tandem repeats. Evidence in *Drosophila* and plants suggests that

somatic pairing may occur between homologous sequences resulting in a nucleation signal for inactivation.[22] Unfortunately, homologous recombination by electroporation, resulting in singly copy integration of foreign DNA, is also susceptible to similar inactivation.[96]

Transgenic Mice

The use of transgenic mice provides a powerful molecular biological tool in which to analyze the consequences of integration of DNA fragments into the mouse genome. Transgenic mice have been used to analyze tissue specific and developmental patterns of gene expression, and the consequence of over expression and mutation of certain genes. It is becoming evident however that uniform and reproducible transgene expression is difficult to achieve. Mosaic and aberrant expression, and varying expression between similar lines of transgenics are common. Factors which may influence expression in transgenic mice include chromosomal position, copy number, cis-acting elements, and genetic modifiers.[21,64]

The use of transgenic mice offers the advantage of analyzing parent of origin effects or imprinting. Transgenes can be transmitted through one germline independent of the other, examined at hemizygous loci, used to probe for imprinted genes by virtue of position effects [20,92], or to identify cis acting elements of transgenes which exhibit parental effects independent of position [13,90,101].

Many features of endogenously imprinted genes resemble imprinted transgenes. Imprinted transgenes show parent specific expression patterns, transformation of parent specific imprints upon passage though the germline of the opposite sex, and correlation between hypomethylation and expression. [13,85,91,101,106]

Chromosomal Position Effects

There are two types of position effects; stable and variegating.[22] Transgenes that integrate close to heterochromatin may be silenced in a random fashion, resulting in mosaic expression. Variegating position effects have been documented for transgenes that integrate into the X chromosome.[19,43] Stable position effects occur when transgenes are modulated by enhancer or repressor elements located in the neighborhood of the integration site, and is usually associated with integration into euchromatin. Essentially, transgenes act like enhancer traps.[15] Such integrations give rise to expression patterns which are reproducible between individuals in a given line.

Copy Number

The notion that more transgenes equals more expression does not hold when dealing with transgenic mice. In general, copy number is inversely correlated with overall expression and decrease in expression is associated with hypermethylation.[73] However, heterogeneous expression was not addressed, and thus single or low copy integrations may still be susceptible to inactivation, consistent with variegation in single copy integrations in ES cells.[96]

Cis-Acting Elements

Specific DNA sequences when included in plasmid constructs can confer position independence and/ or copy number dependence by insulating the transgene from neighboring DNA effects. Scaffold-attachment regions (SARs) or Matrix-attachment regions (MARs) are AT rich sequences approximately 0.5-1.0kb in length often flanking regulatory regions of translated sequences. They were originally identified by fractionation of the chromosome scaffold in vitro, and serve to anchor DNA to the nuclear matrix.[45,84]

Transgenic mice harboring a transgene driven by the whey acidic promoter was prone to severe position effects. Addition of MARs flanking the transgene resulted in position independent expression.[72]

Locus control regions (LCRs) are cis elements which are associated with the initiation and/ or maintenance of a cell-type specific open chromatin configuration. (-globin transgenes are susceptible to variable and position dependent expression. Inclusion of the (-globin LCR confers both position independent and copy number dependent expression.[74]

Strain Specific Modifiers

Variegation of transgene expression may be affected by genetic background. Expression and/ or function of chromatin factors may differ between different inbred strains, and may influence expressability of a transgenes. Back crossing into a different genetic background can modify transgene expression patterns.[64] The DBA/2 genetic background is often associated with transgene demethylation whereas C57BL and BALB/c strains are associated with de novo methylation [3,27].

The Pgk-1,2-LacZ Transgenic Mouse

Pgk-1 gene is an X-linked gene encoding 3-phosphogycerate kinase (PGK). PGK is a 45 kD protein involved in energy metabolism through the glycolytic pathway and also functions as a co-factor for DNA polymerase ([42]. It is expressed in all tissues and cells, except for sperm, where an autosomal Pgk-2 gene is replaces its expression.[70]

The Pgk-1 gene is ubiquitously expressed and its promoter has been used to drive the expression of many genes.[66,69] The murine Pgk-1 promoter is particularly active when used to drive reporter genes in embryonal carcinoma cells.[68]

A 304bp upstream activator sequence regulates the expression of the Pgk-1 promoter. Expression in undifferentiated cells is coincident with two of three Dnase hypersensitive sites. A factor, present only in differentiated cells, binds the UAS and may be responsible for decreased expression.[100]

Transgenic mice carrying the Pgk-1 promoter driving the E. Coli LacZ gene shows widespread expression of the transgene. It is carried on an autosome and is not inactivated in female tissues. The transgene is expressed in most tissues with a reproducible degree of cellular variation or mosaicism.[69]

The Bacterial E. Coli LacZ gene

Bacterial β-galactosidase or LacZ has been used as a reporter gene because of the ease of product detection in assays and in histological sections. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) is a commonly used substrate for the detection of lacZ protein. The end product of the enzymatic reaction is a blue precipitate which is visually detectable. The use of LacZ provides a powerful tool to study gene expression. However, there is increasing evidence that LacZ contains cis-acting elements that may influence gene expression.

The 3-hydroxy-3-methylglutaryl (HMG) gene is ubiquitously expressed in all tissues.[60] The HMG promoter driving Chloramphenicol Acetyltransferase (CAT) also directed expression in all tissues tested. [73] However, fusion of the LacZ gene to the same HMG regulatory sequences resulted in only testis specific expression.[78]

The Neomycin gene driven by the human cytomegalovirus (CMV) immediate early protein promoter resulted in expression in various tissues.[93] Fusion with LacZ abrogated ubiquitous and uniform expression.[50]

The fusion of the promoter of the class I major histocompatibility complex (H-2Kb) with LacZ gave rise to unexpected and unusual expression patterns.[15] The same promoter driving a human growth hormone drove expression in all tissues examined.[76]

LCRs and SARs are capable of insulating genes from chromosomal effects. LCRs confer stable expression onto globin transgenes. Transgenic mice carrying LacZ driven by a α-globin promoter flanked by an LCR, lose position independent expression.[32] The capacity of SARs to insulate genes is also absent in a c-kit/LacZ fusion transgene.[18]

Lastly, a fusion of the human keratin 18 gene with lacZ resulted in parental specific transmission of the transgene. Maternal inheritance conferred proper expression in the liver whereas paternal inheritance superimposed ectopic expression in retina and mesodermal tissues. A recessive lethal phenotype, with no parent of origin effects, was revealed when mice were bred to homozygosity. DNA methylation of the LacZ gene correlated with gene inactivation.[106]

Project Objectives

The objective of my research was to investigate the phenomenon of transgene inactivation. The study of transgene inactivation is pertinent to both natural phenomena of gene inactivation as well as to the future of gene therapy and research in mammals.

My thesis project is composed of three distinct aspects. The first was to investigate and characterize an unusual strain of Pgk-1,2-lacZ transgenic mice which exhibited characteristics of genomic imprinting. Secondly to characterize of the stability of single copy transgene integrations using targeted disruptions of the Clk/ Sty gene in embryonic stem (ES) cells and in mice. and thirdly to address the nature of transgene inactivation in P19 EC cells.

Towards this end. my research began with an investigation of tissue and cellular expression of the Pgk-1.2-lacZ transgene passaged through both parental germlines. Methylation was targeted as a candidate process responsible for gene inactivation. We performed methylation analysis of different regions of the transgene and used demethylating agents on non-expressing transgenic Mouse Embryonic Fibroblasts (MEF). Furthermore. additional transgenics were constructed to recapitulate properties of the single unusual line. My experiments with these founders included histological and methylation analyses. The results of these studies are presented in chapter 1.

The instability of transfected genes has been largely attributed to the tandem nature of transgenes. Generally expression is correlated with lower copy numbers. We were presented with the opportunity to investigate this aspect of transgene regulation using ES cells and mice carrying a targeted disruption of the Sty/ Clk gene. Homologous recombination into cells results in single copy single integration. My investigation included

the characterization of expression of an ires-(geo cassette in ES cells and in mouse tissues, and a determination of the nature of inactivation in these two systems. The results of these studies are presented in Chapter 2.

Transfection of genes into EC cells results in gene loss and gene inactivation. We sought to investigate whether methylation was involved in the process of inactivation in EC cells. To do this, we performed methylation analysis on the transgene using different methylation sensitive enzymes, and treated non-expressing cells with demethylating agents to reactivate expression. The results of these studies are presented in Chapter 3.

Chapter 1

Abstract

One line of Pgk-1,2-lacZ transgenic mice is prone to unusual germline specific effects. Maternal transmission of the transgene results in inactivation of the transgene in all tissues except for brain, testis, and heart. Furthermore, the maternal imprint imposed upon the transgene is irreversible despite retransmission through the male germline. We sought to characterize this unusual line by northern and histological analysis, and to determine if methylation was involved in inactivation of the Pgk-1,2-lacZ transgene.

HpaII sites are heavily methylated within the lacZ region of the transgene in kidney and testis, regardless of parental transmission, or status of tissue expression. Slight under methylation is only evident within expressing tissues, suggesting that a small proportion of the transgene array may remain undermethylated, and that these may be responsible for expression within positive cells. Treatment of mouse embryonic fibroblasts carrying maternally inherited transgenes, with 5-aza-2-deoxycytidine, reactivates lacZ expression. Taken together, these results correlate inactivation of Pgk-1,2-lacZ with methylation.

Additional Pgk-1,2-lacZ transgenics were generated to recapitulate the unusual effects of the single line. Methylation Analysis of DNA from founder animals revealed that the lacZ regions was also methylated and was established sometime during development. Histological analysis of various tissues within founder animals revealed extensive variability in levels and patterns of tissue expression, suggesting that Pgk-1,2-lacZ was prone to position effects. Matings are still underway, to determine if imprinting effects can be duplicated in the new transgenic lines.

Introduction

Genomic imprinting in mammals is a reversible epigenetic modification of DNA that occurs as a result of parental inheritance. It is a gametic mark that specifies differences between paternal and maternal genomes. The result is preferential expression of one allele over another. Non-viability of androgenetic and gynogenetic embryos first suggested a difference between parental genomes.[71] Translocations resulting in uniparental disomies led to the identification of imprinted chromosomal regions.[10]

Methylation is thought to be implicated in the mechanism of genomic imprinting.[11,12,57] It is the only known form of epigenetic modification that is heritable and reversible, and has been correlated with the formation of heterochromatin.[36]

The first imprinted gene to be characterized was the Igf2 gene. Paternal inheritance of IGF-2 results in its expression, and is silenced by maternal transmission. The H19 gene is located within close proximity to IGF-2, but is oppositely imprinted, and is only expressed upon transmission through the female germline.[19] Expressivity of genes can also be affected by parental inheritance and have been documented for some human diseases such as Huntington's chorea.[87]

The Pgk-1 promoter is capable of driving the expression of exogenous genes in various cell lines and in transgenic mice. Transgenic mice carrying the Pgk-1 promoter driving E. Coli lacZ showed widespread distribution but not uniform transgene expression. Transgenics were created by microinjection into the pronuclei of F1 (C57BL/ C3H) zygotes and three founders identified. The founders were maintained by crossing male transgenics with C3H/ He females.[69]

The transgene was carried on autosomes and was not inactivated during spermatogenesis or X chromosome inactivation in female cells. The lack of imprinting was attributed to the absence of cis-acting elements present on the X chromosome. No identifiable differences in transgene expression were noted regardless of parental inheritance.[69]

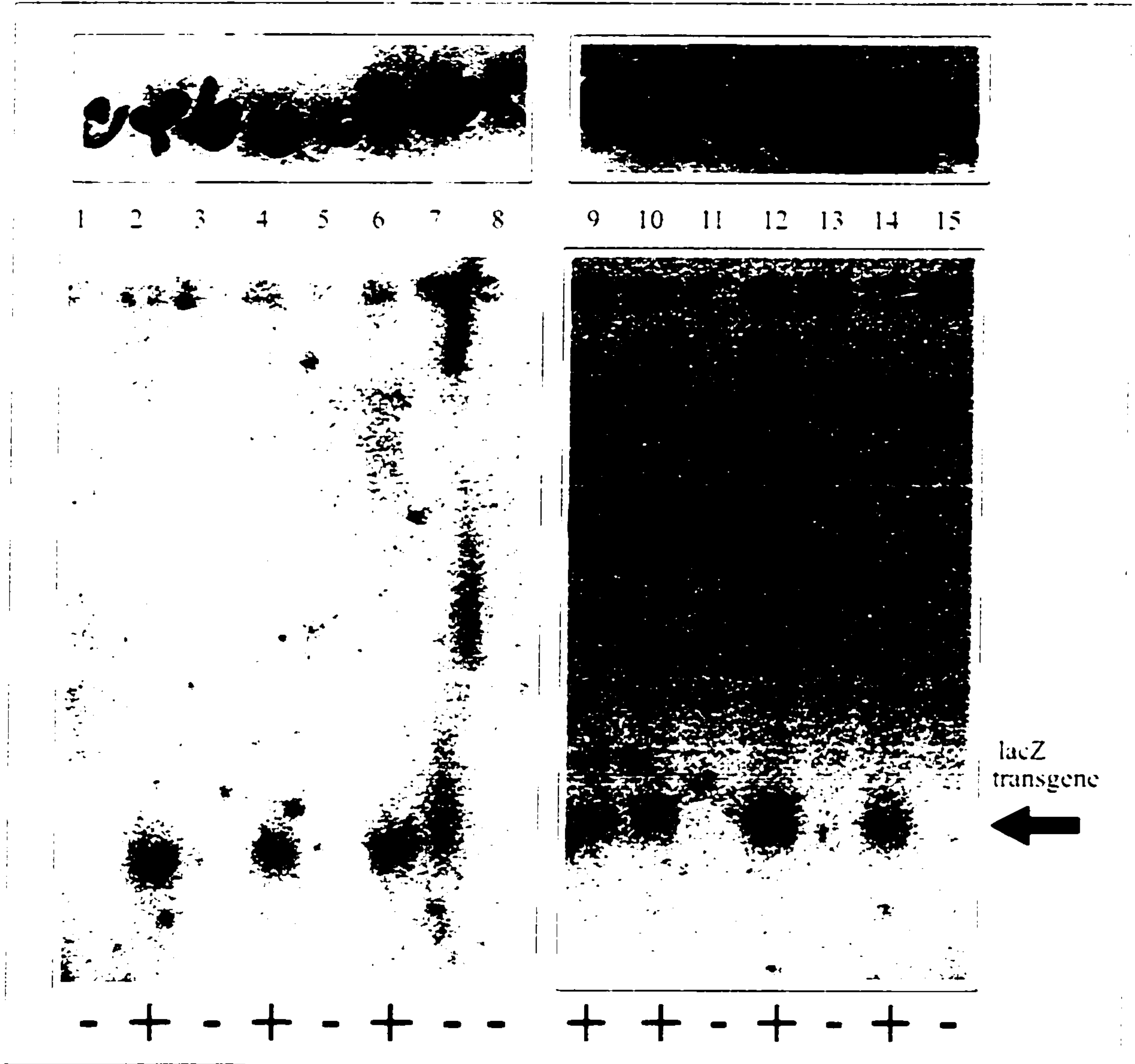
One line of Pgk-1,2-lacZ transgenic mice was maintained for further study and bred to homozygosity for easier maintenance. During the course of investigating various aspects of this transgenic mouse line, we found that this line was subject to imprinting phenomena.

Transgenic mice were originally phenotyped by the virtue of expression within tail clippings from 21 day old mice. Blue tails was a reliable gauge of transgene transmittance. Passage of the transgene through the female germline resulted in loss of expression from tail clips while paternal transmittance retained expression.

Embryos carrying paternally transmitted transgenes expressed lacZ (Fig 1 lanes 2, 4, 6: Experiments for this figure were done by Karen Jardine and Dr. Michael W. McBurney) whereas embryos carrying maternally transmitted transgenes inactivated lacZ expression (Fig 1 lanes 9, 10, 12, 14). We sought to further characterize the characteristics of this unusual Pgk-1,2-lacZ transgenic line.

Figure 1: The Pgk-1,2-lacZ transgene is inactivated upon transmission through the female germline

Day 9 mouse embryos were isolated from matings between expressing transgenic males X wild type females (**lanes 1-8**) and from expressing transgenic females X wild type males (**lanes 9-15**). Embryos were fixed in 0.2% glutaraldehyde and stained with X-gal. Subsequently, DNA was isolated from stained embryos and digested with EcoRI. Restriction fragments were separated by electrophoresis, blotted and hybridized with a lacZ probe. Embryos that which inherit the Pgk-1,2-lacZ transgene can be identified by the presence of a 3.0kb band in southern analyses. The lacZ probe hybridized to seven DNA samples (**lanes 2, 4, 6, 9, 10, 12, 14**) which identify these embryos as transgenics. In embryos with paternally inherited transgenes, the transgene is expressed (**2, 4, 6**). In embryos with maternally inherited transgenes, the transgene is inactivated (**9, 10, 12, 14**).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lacZ
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Materials and Methods

Probes

Probe DNAs were prepared and used as follows. Briefly, plasmid DNA was digested using appropriate restriction endonucleases under the conditions recommended by manufacturer. DNA was separated by electrophoresis through 1% low melt agarose gels in 0.1X TE. The appropriate DNA fragments were isolated by through cuts into the agarose gels which were recovered by dissolving gel fragments in TE at 55°C, extracted with phenol-chloroform (1:1), and finally redissolved in TE. The LacZ probe was a 2.3kbp EcoRI-ClaI fragment from pDM2 and the α-tubulin probe was a full length mouse cDNA. Probe DNAs were 32P-dCTP labeled using the standard multiprime DNA labelling protocol (Amersham).

DNA Isolation and Electrophoresis

Genomic DNA was extracted from adult mouse tissues and mouse embryonic fibroblasts similarly to previously described methods (194). DNA was extracted from adult mouse organs by placing tissues in ice cold PBS, mincing the tissues using surgical blades, and then incubating the minced tissues in lysis buffer (0.2% SDS, 200mM NaCl, 5mM EDTA, and 100mM Tris HCl (pH 8.5) containing 100(g/mL proteinase K), at 37°C overnight with gentle agitation. One volume of isopropanol was added to recover DNA from the lysate. The DNA was dissolved in TE (10mM Tris HCl (pH 8) and 1mM EDTA).

Purified genomic DNA was digested by restriction endonucleases at a concentration of 10U/ μg. Digested genomic DNA was separated by electrophoresis through 1.0% agarose gels in 0.1X TE. Separated DNAs were transferred to Hybond-N membranes (Amersham) and treated with ultraviolet light at 110mJ using a GS Gene linker UV chamber (Biorad).

Northern Blot

RNA was isolated using TRIzol (Gibco BRL). Briefly, adult mouse tissues were homogenized in TRIzol. Following incubation at room temperature for 5 minutes 0.2mL chloroform/ mL TRIzol was added to the sample and incubated for an additional 2 minutes. Samples were centrifuged at 12,000g for 15 minutes at 4°C and aqueous phase mixed with 0.5mL isopropanol/ mL TRIzol. Following a 10 minute incubation at room temperature, samples were centrifuged at 12,000g for 10 minutes at 4°C. After washing with 70% ethanol, the RNA was dissolved into RNase-free water.

RNA was separated by electrophoresis through 1% agarose gels in 20mM 3-[N-morpholino]propane-sulfonic acid, 1mM EDTA, 5mM sodium acetate at pH 7, and 10% formaldehyde. Separated RNAs were transferred to Hybond-N membranes (Amersham) and treated with ultraviolet light at 110mJ using a GS Gene linker UV chamber (Biorad).

Slot Blot

Purified DNA was dissolved in 400(L 10mM TE at pH 7.0, to which 0.1 volume of 3.0M NaOH was added. Solutions were heated at 65°C for 1hr (destroy RNA and denature DNA) and subsequently neutralized with 1 volume of 2.0M ammonium acetate (NH_4OAc). DNA samples were loaded on a minifold II device as specified (Schleicher and Schuell Inc.) and each sample was loaded 3 times in decreasing amounts (50% vol., 10% vol., and 5% vol.) under suction. DNA was crosslinked to Hybond-N membranes (Amersham) and treated with ultraviolet light at 110mJ using a GS Gene linker UV chamber (Biorad).

Probe Hybridization

Prehybridization was carried out in 50% deionized formamide, 5 x SSPE, 2.5 x Denhardt's solution, 0.1% SDS, 0.2mg/mL denatured salmon testis DNA (1 x SSPE is 0.15M NaCl, 10mM $Na_2H_2PO_4$, and 1mM EDTA). Membranes were hybridized for 15-24 hours at 42oC in prehybridization solution containing 32P-dCTP labeled probes. Following incubation, membranes were washed once in 2 x SSC and 0.1% SDS at 42°C for 15 minutes, followed by several changes of 0.2 x SSC and 0.1% SDS at 65°C for 45 minutes. Radioactivity was visualized and analyzed using Molecular Dynamics Phosphorimager SI.

Histological Analysis

Mouse tissues were prepared from animals that were anesthetized and perfused through the heart with PBS followed by Lanas fixative (4% paraformaldehyde and 0.2% saturated picric acid in 0.16M sodium phosphate buffer at pH 6.9). Tissues were removed, postfixed for 90 minutes and transferred to 10% sucrose in 100mM phosphate buffer. Tissues were frozen and 12(m cryostat sections were cut, thaw mounted onto uncoated slides (Superfrost Plus, Fisher Scientific), stained with X-gal, and counterstained with either nuclear fast red or Harris hematoxylin for 2-3 minutes before mounting.

Cell Culture

Mouse embryonic fibroblasts were prepared from 12-13 day old mouse embryos from transgenic homozygous females or heterozygous females X wild type males. Briefly, embryos were isolated and disaggregated by fine mincing with sterilized surgical blades. Cells were cultured in minimum essential medium alpha (MEM() (Gibco) supplemented with 15% fetal bovine serum (Cansera International Inc). The cells were maintained in

plastic tissue culture dishes, which were placed at 37°C in a 5.0% CO2 incubator. They were kept in exponential growth phase by routinely sub-culturing cells at intervals of approximately 96 hours. Briefly, the cell monolayer was washed in phosphate-buffered saline (PBS) (0.8% NaCl, 0.2% KCl, 0.02% KH2 PO4 and Na_2HPO_4), and then incubated in trypsin-EDTA (1mM EDTA and 0.025% trypsin in PBS) for 10 minutes. The detached cells were dispersed by vigorous pipetting. They were then counted and replated at a density of 150cells/mm2 into fresh medium.

Cells were exposed to various concentrations of 5-Aza-2-DeoxyCytidine (5A2C) essentially as described (30). Cultures were grown in the presence of various concentrations of 5A2C for approximately two cell cycles (approximately 48hr), after which the drug was removed and the cells were washed and incubated for an additional 48 hours. Subsequently, cells were fixed in 0.2% glutaraldehyde in PBS for 1 hour, washed and incubated overnight at 37oC in X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside).

Immunofluorescence

Frozen tissue cryosections were thaw mounted onto uncoated slides (Superfrost Plus, Fisher Scientific), air dried for 30min at room temperature, and washed 3X in PBS for 30min. Monoclonal J1E7 mouse anti-(-galactosidase antibodies were added to PBS with 0.03% triton X-100 and incubated overnight at 4°C. Glass slides were washed 3X in PBS for 30min before the addition of CY3-conjugated goat anti-mouse antibody, and incubated for 1hr in the dark at room temperature. Proceeding incubation glass slides were rinsed carefully 3X in PBS for 15min, after which they were mounted with 90% glycerol. Staining was visualized with a Zeiss Axiophot microscope.

Results

Tissue Specific Expression Depends Upon Parental Inheritance

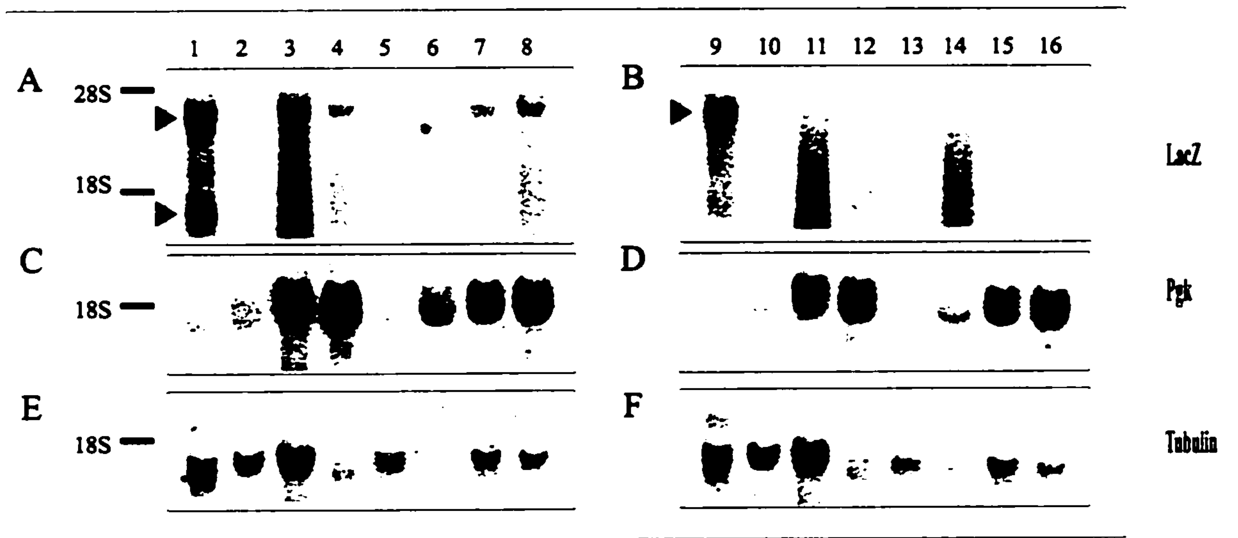
Northern analysis of RNA isolated from various tissues from adult mice derived from a transgenic mother or father revealed a differential lacZ tissue expression pattern (Fig 2). Paternally derived mice expressed the transgene in the testis, brain, calf muscle, liver, heart, and kidney (Fig 2A), consistent with previously published results (12). Maternally derived mice lost expression in all tissues tested except for testis (Fig 2B). Both paternally and maternally inherited transgenes were expressed in the testis (Fig 2A,B). However, testicular expression in paternally transmitted transgenes results in the production of two RNA species, 3.0kb and 1.5kb (Fig 2A lane1). The 1.5kb transcript is absent from the testis of males derived from transgenic females (Fig 2B lane9) and is thought to be a splice variant of the lacZ gene.

A more detailed examination of transgene expression was assessed by histological analysis. Northern analyses give an average of global cellular expression within a given tissue and does not address the question of heterocellular expression. Mosaicism is a common feature of transgene expression and the extent of mosaicism may render transgene expression undetectable.

Histological analysis of various tissues from over 10 male and female adult animals derived from transgenic C3H/ HE females were assessed. Additionally, histology and northern analyses were performed on adult mice derived from up to 5 generations of breeding into a CD1 background. There were no detectable differences in tissue or cellular expression between the two different genetic backgrounds.

Fig 2: Northern analysis of transgene expression in Pgk-1,2-LacZ^{on} and Pgk-1,2-LacZ^{off} mice

RNA was isolated from various tissues of male adult animals of Pgk-1,2-LacZ^{on} mice (**A, C, E**) and Pgk-1,2-LacZ^{off} mice (**B, D, F**), with the exception of mammary tissue RNA which was isolated from female mice. These RNAs were electrophoresed, blotted and hybridized to a probe for lacZ (**A, B**), Pgk-1 (**C, D**), and tubulin (**E, F**). Tissues are denoted as testis (**1,9**), mammary gland (**2,10**), brain (**3,11**), calf muscle (**4,12**), spleen (**5,13**), liver (**6,14**).heart (**7,15**), and kidney (**8,16**). Arrows indicate lacZ transcripts.



1 2 3 4 5 6 7 8
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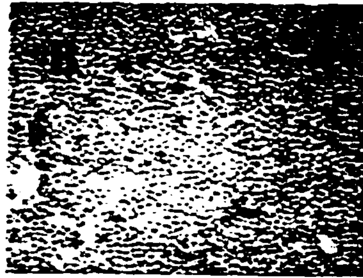
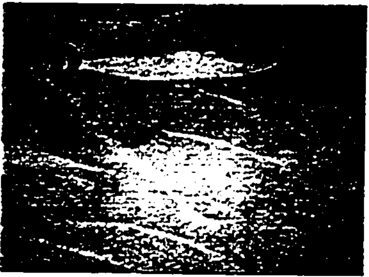
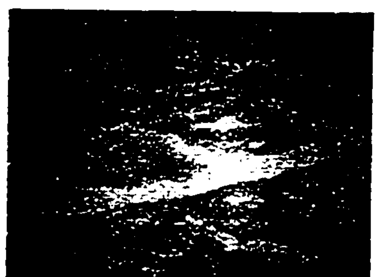
18S
LacZ
Pgk
Tubulin

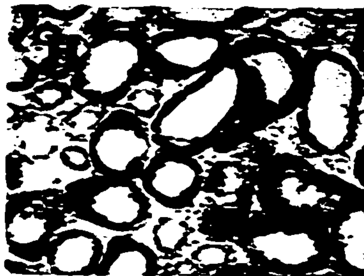
Fig 3: Histological analysis of lacZ expression from various tissues from Pgk-1,2-LacZ^{on} and Pgk-1,2-LacZ^{off} mice

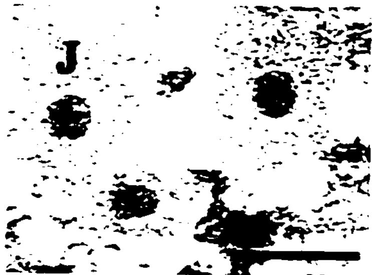
Panels **A, D, G, J, M** are tissue cryosections from Pgk-1,2-lacZ^{off} mice. **Panels B, E, H, K, N** are tissue cryosections from Pgk-1,2-lacZ^{on} mice and panels **C, F, I, L, O** represent cryosections from wild-type mice. All sections are 12µm thick. stained with X-gal. and counterstained with nuclear fast red. Expression is heterogeneous in tissues which express the transgene (**E, H, K, M, N**). Tissues from Pgk-1,2-lacZ^{off} mice have lost expression in muscle (**D**). kidney (**G**). and liver (**L**). with the exception of the heart (**M**) where expression is decreased. Negative controls show no X-gal staining (**C, F, I, L, O**). Spleen (**A, B, C**). calf muscle (**D, E, F**). kidney (**G, H, I**). liver (**J, K, L**) and heart (**M, N, O**) are represented. The bar in panel **A** is 10µm and represents all panels with the exception of panels **J, K, L** in which the bar represents 2µm.







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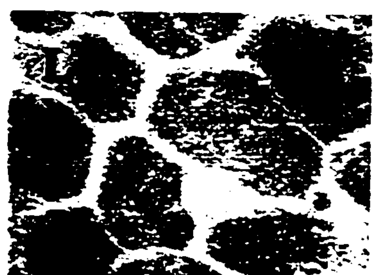
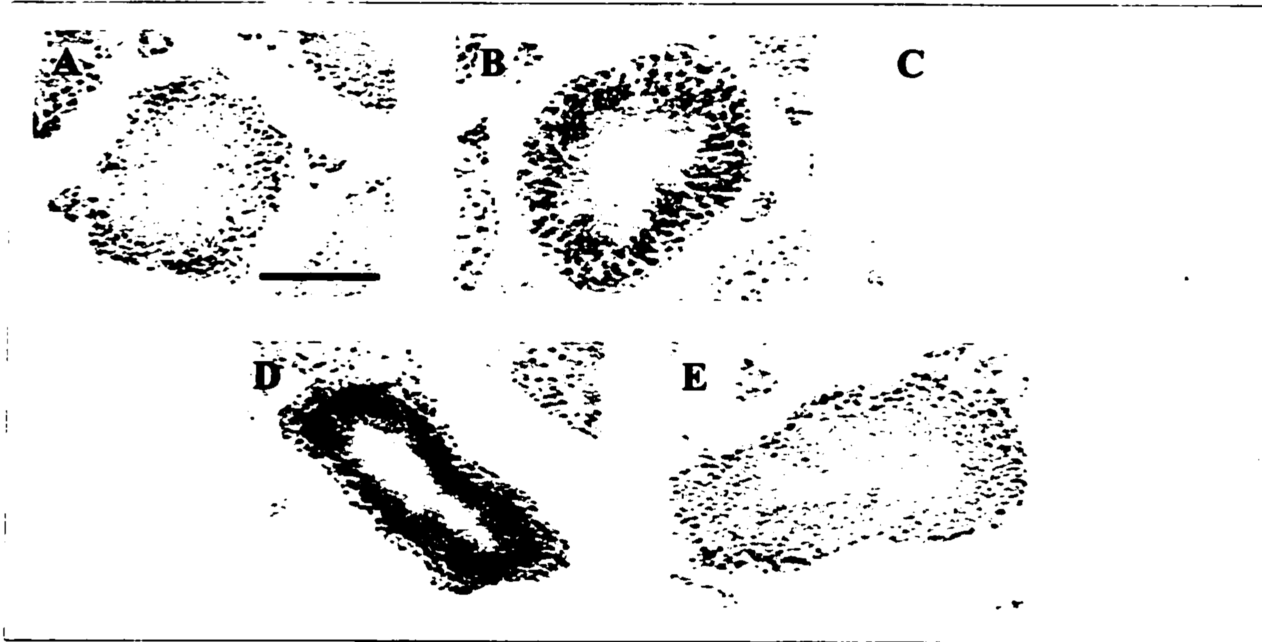


M

Figure 4: Transgene expression in the testis of Pgk-1,2-lacZ^{on} and Pgk-1,2-lacZ^{off} mice

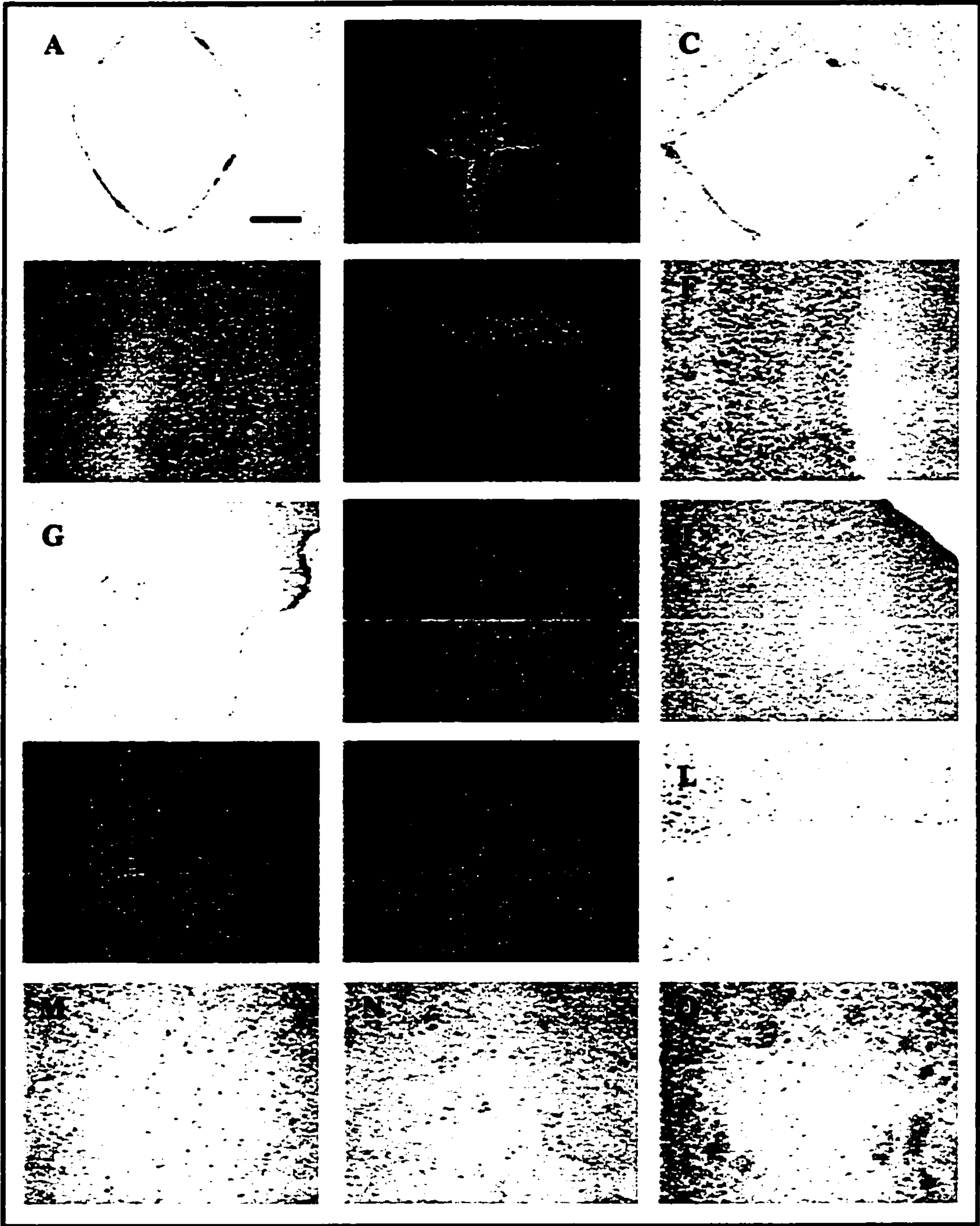
Testes were isolated from transgenic males carrying either maternal or paternal inherited transgenes, and from wild type males. 12µm testis cryosections were cut, stained with X-gal, and counterstained with nuclear fast red. The testis of both Pgk-1.2-lacZ^{on} (**panels D, E**) and Pgk-1.2-lacZ^{off} (**panels A, B**) mice express the transgene and appear to have similar stage specific expression patterns. Non transgenic adult mouse testis (**panel C**) show a little staining. If staining is present it is localized only to leydig cells and not germ cells. The bar in **panel A** is 10µm



A
B
C
D
E

Figure 5: Transgene expression in the brain of Pgk-1,2-lacZ^{on} and Pgk-1,2-lacZ^{off} mice

Brains from adult mice were frozen and 12μm sections cut. Sections were X-gal stained and counterstained with nuclear fast red, before mounting. Panels **A, D, G, J, M** are tissue cryosections from Pgk-1,2-lacZ^{off} mice. **Panels B, E, H, K, N** are tissue cryosections from Pgk-1,2-lacZ^{on} mice and **panels C, F, I, L, O** represent cryosections from wild-type mice. In Pgk-1,2-lacZ^{on} mice, expression is found within the ependymal cells (**panel A**), cerebellum (**panel D**), cortex (**panel G**), dendate (**panel J**), and caudate putamen (**panel M**). Expression in Pgk-1,2-lacZ^{off} is limited to the ependyma (**panel B**) and to the caudate putamen (**panel N**). LacZ staining is absent from all wild-type brain sections (**panels C, F, I, L, O**). Aquaduct (**panels A, B, C**), cerebellum (**panels D, E, F**), cortex (**panels G, H, I**), dendate gyrus (**panels J, K, L**), and caudate putamen (**panels M, N, O**). The bar in **panel A** is 10μm and represents **panels A-I**. The bar in **panel J** is also 10μm and represents **panels J-O**.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Histology on tissues derived from paternally derived transgenics were consistent with expression data from northern analyses and was also consistent with previously published results (12). Expression was found in calf muscle (Fig 3 panel E), kidney (Fig 3 panel H), liver (Fig 3 panel K), heart (Fig 3 panel N), testis (Fig 4 panels D, E), and brain (Fig 5 panels A, D, G, J, M). Mosaic expression was common to all tissues tested and was reproducible from mouse to mouse. Tissues that carried a maternally inherited transgene showed lacZ activity within the heart (Fig 3 panel M), testis (Fig 4, panel A, B) and brain (Fig 5 panels B, N). The small amount of activity detected within the heart and brain did not parallel results from our northern analysis which was probably due to a limitation of sensitivity. Expression in the brain was present only in ependymal cells (Fig 5 panel B) and cells within the caudate putamen (Fig 5 panel N).

The Transgene is Expressed in The Brain and Reveals a Unique Ventricular Expression Pattern in Maternally Transmitted Transgenics

Serial histological coronal sections were done on brains isolated from transgenic mice carrying maternally inherited transgenes. Expression was localized to ependymal cells lining the ventricular system (Fig 5 panel B) and cells in the caudate putamen (Fig 5 panel N). Histological staining was reconfirmed using monoclonal antibodies directed against E. Coli lacZ (Fig 6).

The ependyma is a constituent of the nervous system consisting of one or more layers, and is composed of ependymal cells, nerve fibers and nerve cells. Ependymal cells line the wall of the brain ventricles and the central canal of the spinal cord, and are

Figure 6: The lacZ protein is detected in the ependyma of Pgk-1,2-lacZ^{off} mice

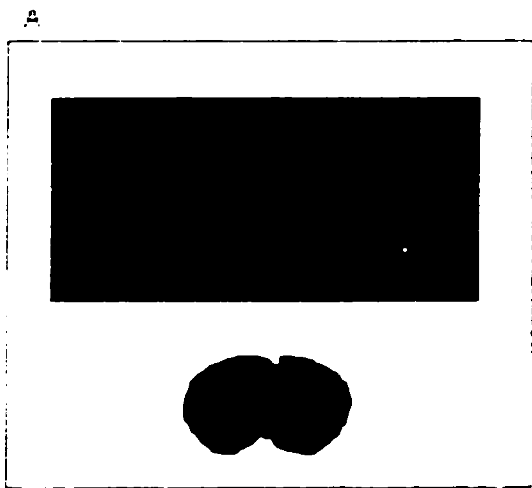
Brains from adult mice were frozen and 12μm sections cut. Sections were incubated with monoclonal J1E7 mouse anti-β-galactosidase antibodies and subsequently with CY3-conjugated goat anti-mouse antibody. The ependyma of the dorsal third ventricle is represented in both panels. **Panel A** is a section from a wild type adult mouse and **panel B** is a brain section from a Pgk-1,2-lacZ^{off} mouse. LacZ protein is detected in the ependyma of Pgk-1,2-lacZ^{off} mice. The bar in **panel A** represents 10μm.

A

B

Figure 7 panels A-P : Unique ventricular expression of the transgene in Pgk-1,2-lacZ^{off} mice

Brains from adult mice were fixed and 12μm serial coronal sections cut. Sections were X-gal stained and counterstained with hematoxylin, before mounting. Panels are arranged in a rostro-caudal fashion. Distances between each coronal section is indicated within each panel in micrometers. Approximate position of enlargements are indicated in accompanying whole brain sections also within each panel. The bar in representative panels is 10μm (**A,E,G,J,M**). Expression starts in the lateral ventricle (**panel A-H**) and continues towards the back of the brain. Symmetry of expression is preserved. A striped pattern of expression becomes more evident within the third ventricle (**panel C-H**). aquaduct (**panel I**) and fourth ventricle (**panel J-P**). Staining patterns are not restricted to ependymal cell types.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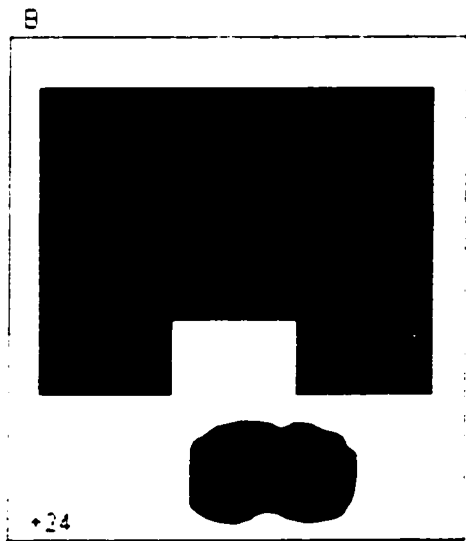
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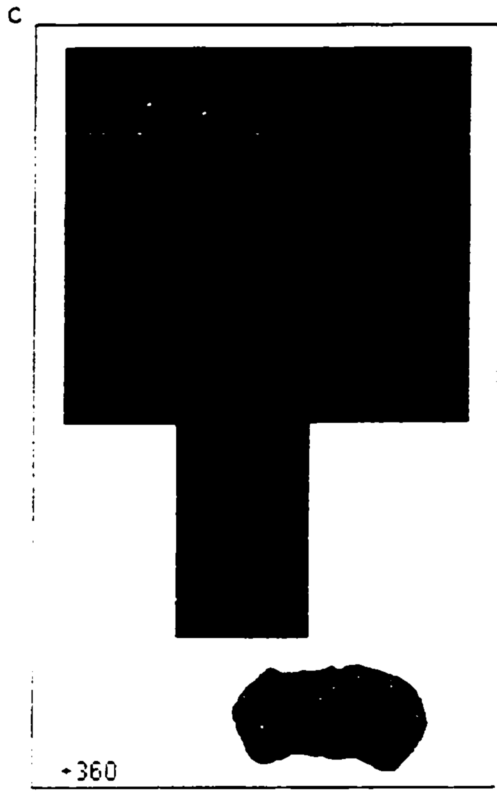
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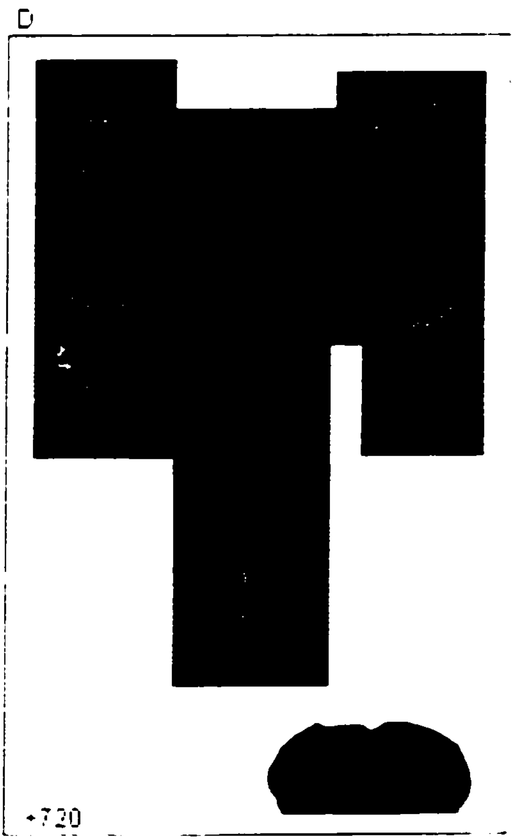
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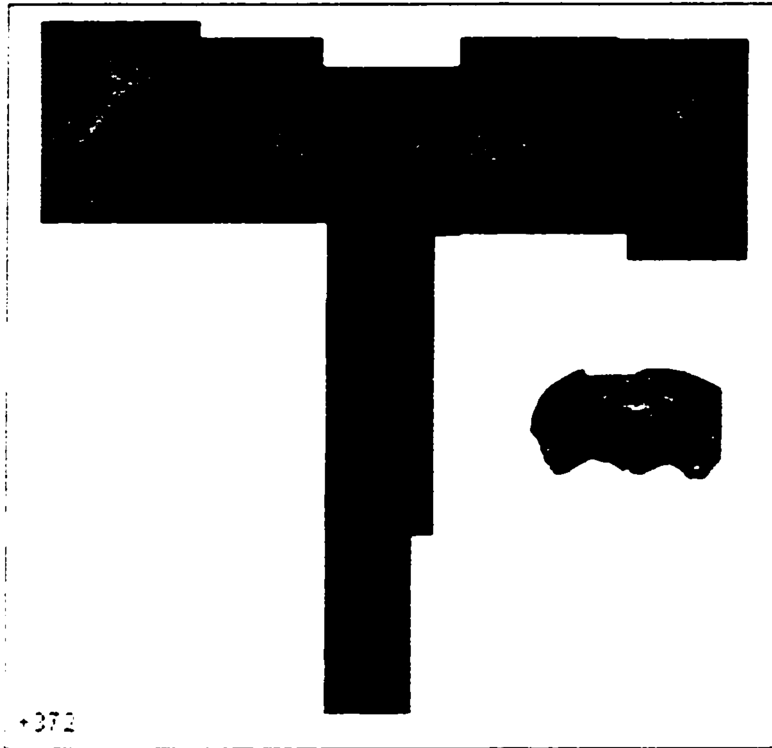

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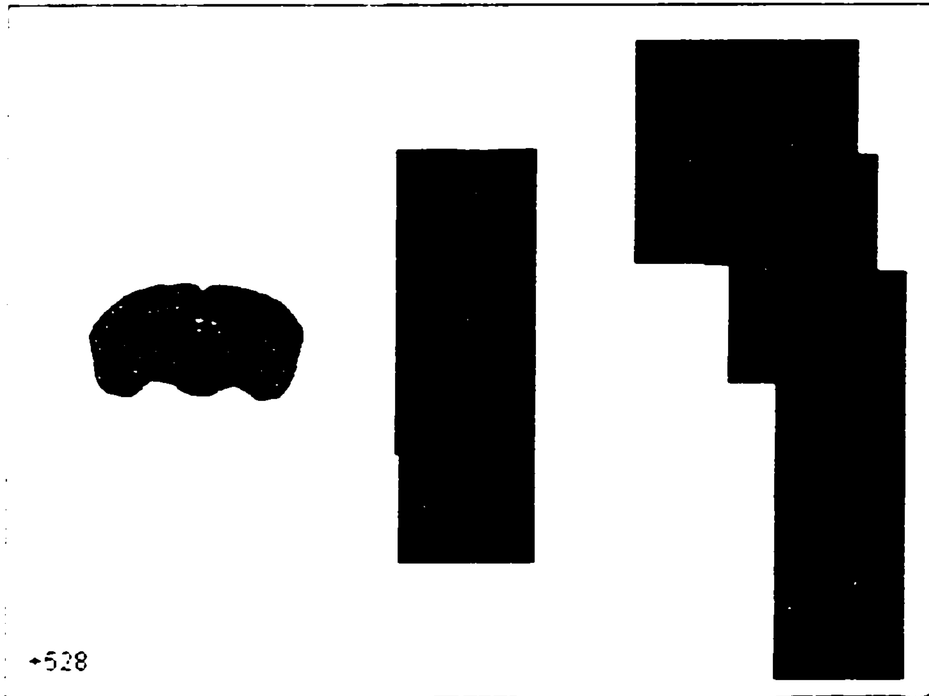

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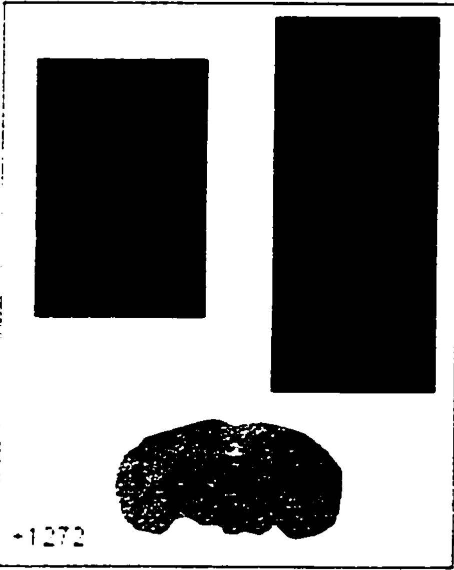

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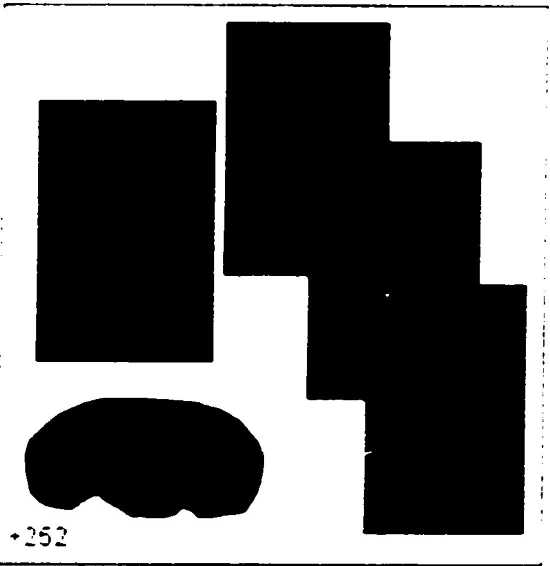

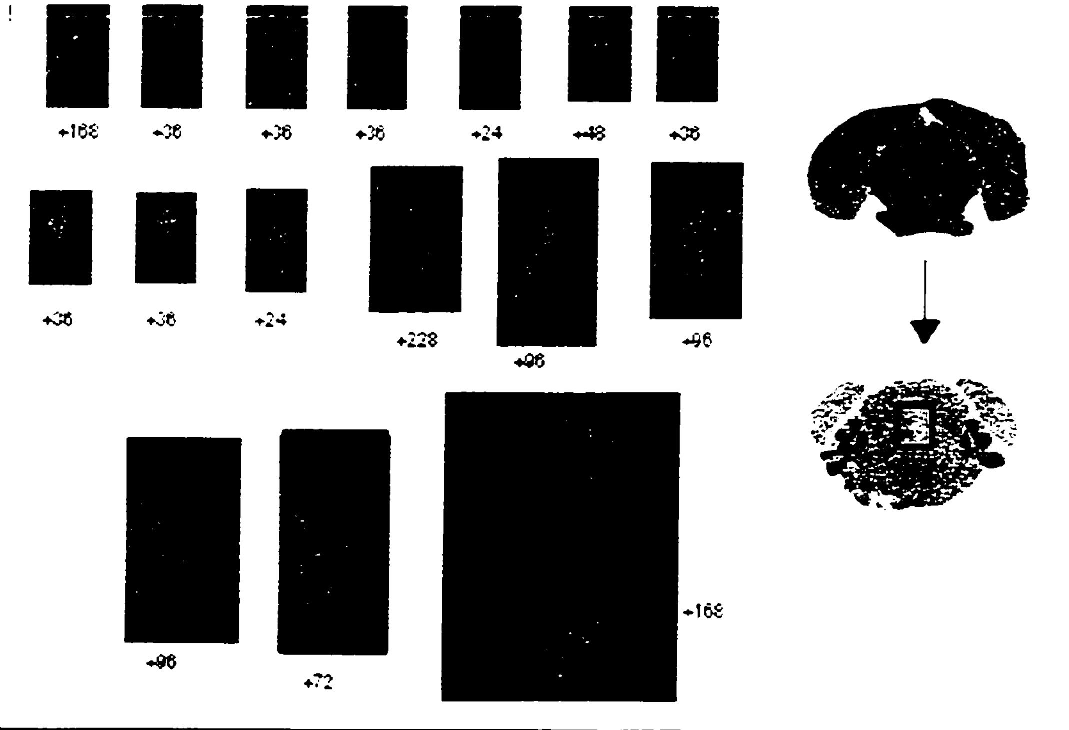

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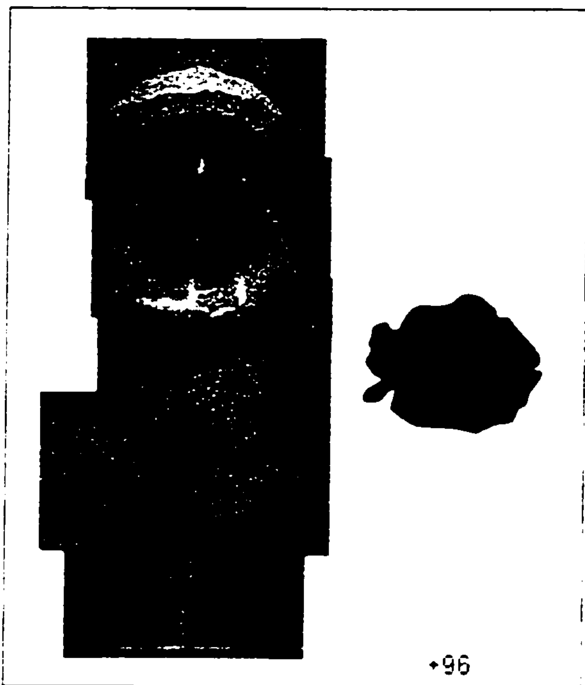
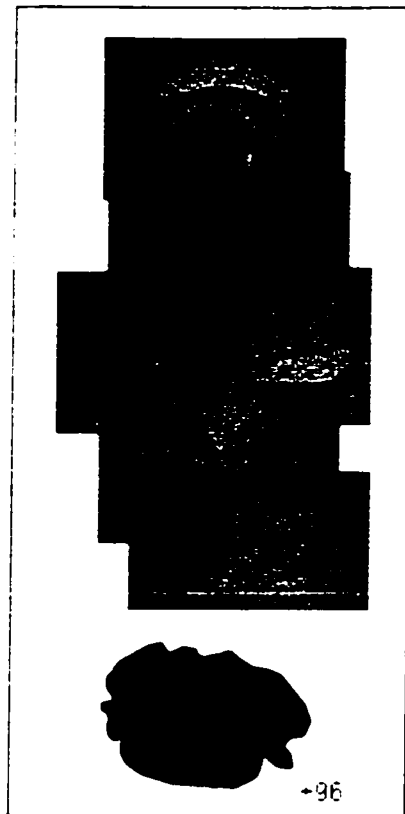


K



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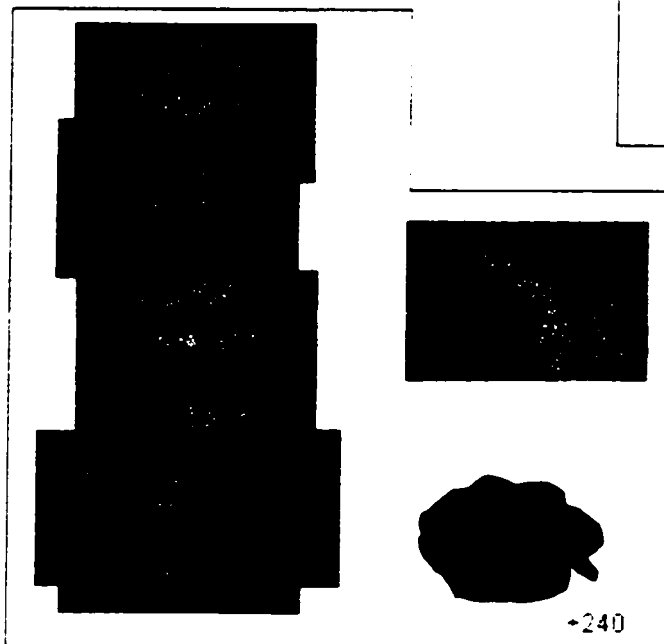

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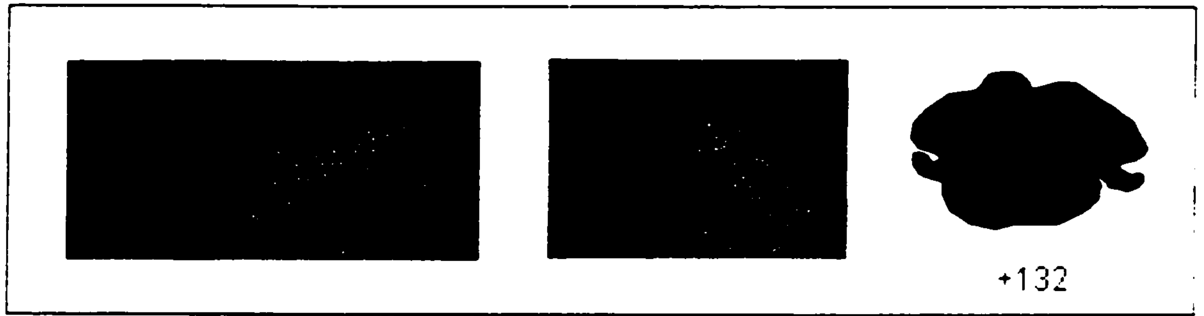

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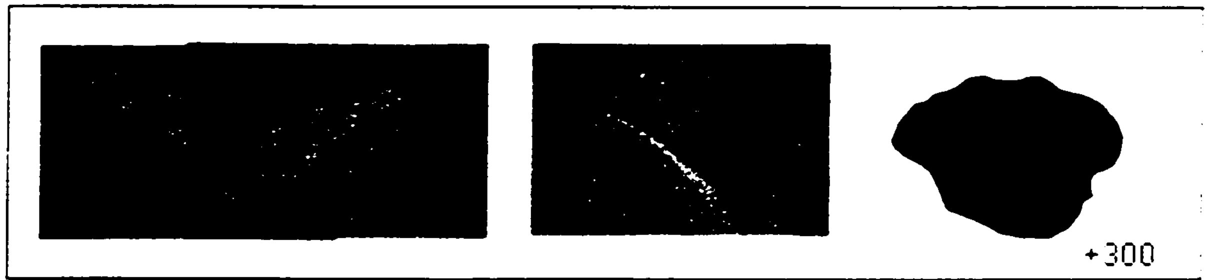
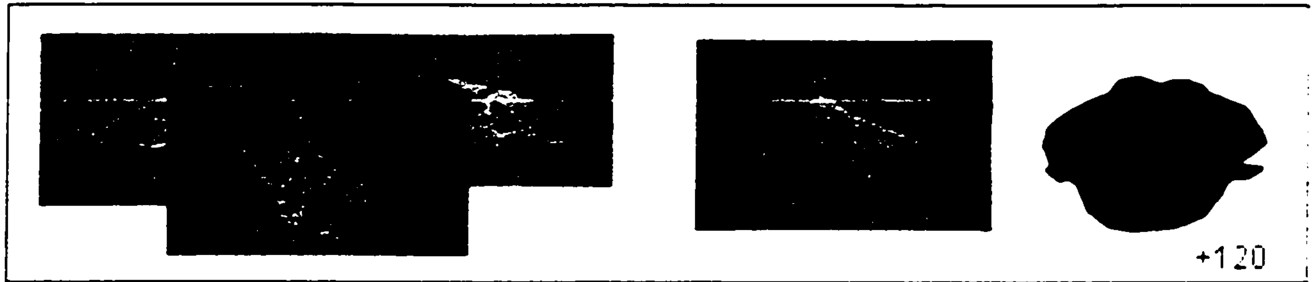
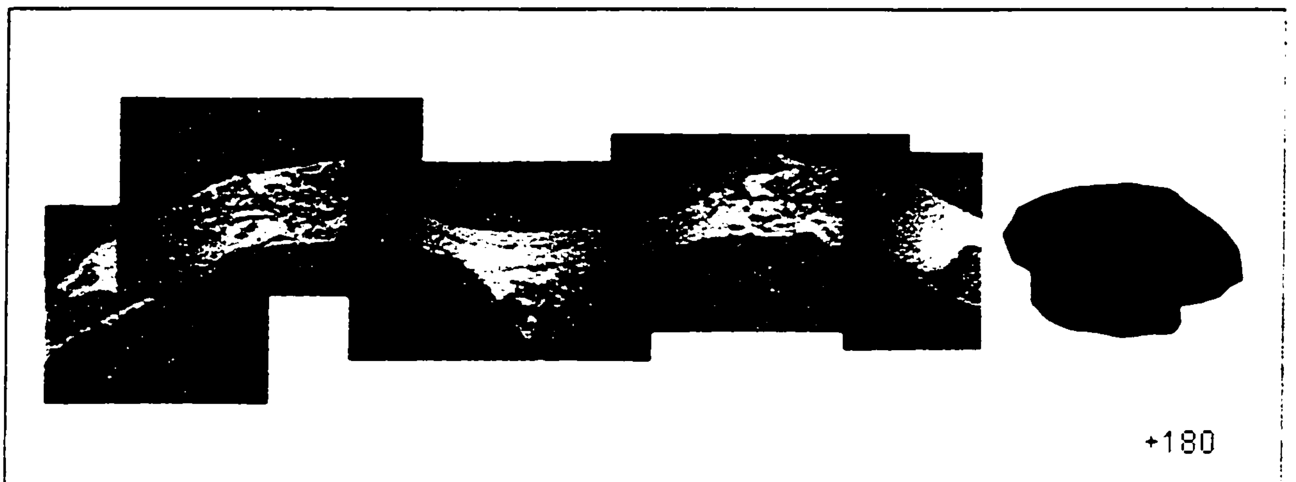


O



P



epithelial like. They have various shapes; flat, cuboidal and collumnar. Some ependymal cells contain basal processes and are termed tanycytes. Tanycytes represent only a minority of ependymal cells most of which lack basal extensions. Our analysis has identified an unappreciated pattern of expression within the ependyma (Fig 7). This pattern of expression is symmetrical throughout the brain and extends from the lateral ventricle to the fourth ventricle. Most of the expression is concentrated within the third ventricle and the aquaduct and manifests itself as a striped pattern. Expression does not appear to be limited to tanycytic or non tanycytic ependymal cells.

The Pgk-1,2-lacZ Gene is Heavily Methylated Regardless of Parental Inheritance or Expression

To determine if methylation was responsible for the unusual characteristics in our mice, we sought to identify methylated regions within the maternally imprinted Pgk-1,2-lacZ transgene that may be correlated with its inactivity. DNA was isolated from kidney and testis of mice carrying either maternally or paternally inherited transgenes. The transgene is expressed in the kidney only if paternally inherited, and thus provides an excellent model tissue to compare expression and methylation. Since testicular transgene expression is common to either parental inheritance, methylation was not expected to be found. DNA was subjected to restriction enzymes EcoRI and MspI or its methylation sensitive isoschizomer HpaII, or with BglII and methylation sensitive XhoI. Complete digestion was assessed by the ability of these enzymes to digest plasmid DNAs in the presence and absence of genomic DNA (Fig 8C and Fig 9C).

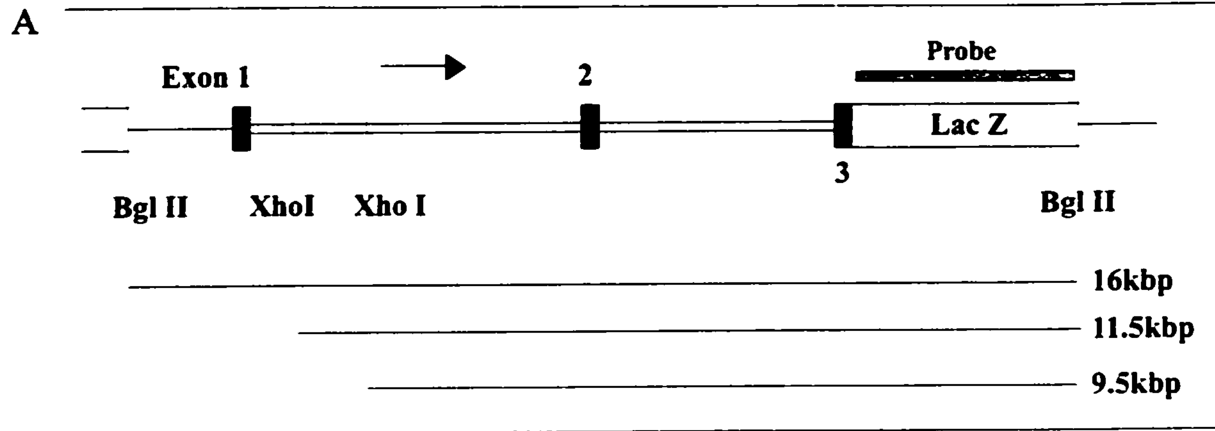
Methylation of intron 1 has been associated with the inactivation of Pgk-1 on the inactive X chromosome [5]. Restriction enzymes BglII and XhoI were used to determine if methylation was present within intron 1 of the Pgk-1,2-lacZ transgene. Restriction sites for BglII are located once in Pgk-1,2-lacZ and Pgk-Puro plasmids yielding a 16kb fragment upon digestion and detection by a lacZ probe. Subsequent digestion with methylation sensitive XhoI depending on unmethylated recognition sites yields three fragments of sizes 9.5, 11, and 16kb (Fig 8A). Southern analysis indicated that the two XhoI sites were unmethylated in all DNAs tested (Fig 8B). No correlation could be found between methylation and inactivation within intron 1.

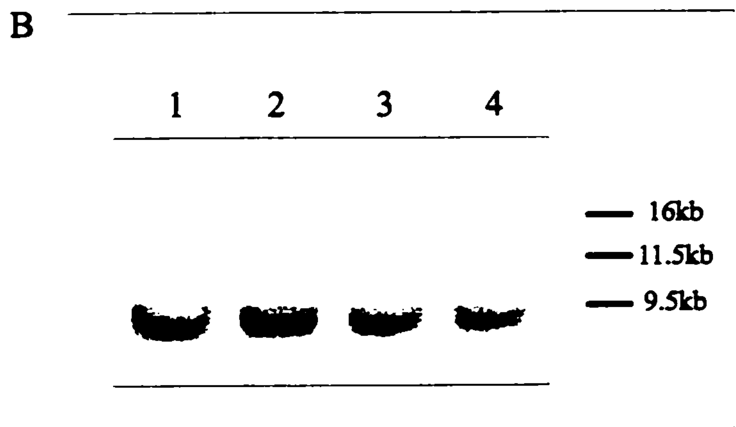
There is increasing evidence that lacZ may contain cis-acting regulatory elements. The methylation of lacZ correlated with parental specific expression of a keratin 18 promoter in transgenic mice.[106] DNAs were digested with restriction enzymes EcoRI and MspI or HpaII. Digested DNAs were separated by electrophoresis, blotted and hybridized to a 2.3kb lacZ probe. EcoRI restriction sites flank the lacZ gene and upon digestion yields a 3.0kb fragment. HpaII is a methylation sensitive enzyme and Msp I, its isoschizomer is methylation insensitive. There are multiple HpaII restriction sites within lacZ. Subsequent digestion of EcoRI digested Pgk-1,2-lacZ with MspI yields many fragments which resolve into three major bands at 700, 350 and 100bp (Fig 9A). HpaII digests yield the same MspI restriction patterns if no methylation is present.

DNAs digested with EcoRI and HpaII yielded a banding pattern which predominantly consisted of a 3.0kb fragment in all samples (Fig 9B lanes 1-8). Only DNA from tissues which expressed the transgene showed additional banding patterns similar to MspI digests (Fig 9B lanes 1-5, 7). These additional bands were weak and

Figure 8: Methylation within the intronic region of Pgk-1,2-lacZ

DNA isolated from the kidney and testis of Pgk-1,2-lacZ^{on} and Pgk-1,2-lacZ^{off} mice and digested with BglII and methylation sensitive enzyme XhoI. Restriction fragments were analyzed by southern blotting. (**A**) A diagram of the Pgk-1,2-lacZ transgene shows restriction sites for BglII and XhoI within the intronic region, along with a reconstruction of possible restriction fragments. The probe used was a 2.3kb lacZ fragment. (**B**) **Lanes 1 and 2** are testis DNA from non-expressing mice and expressing mice, respectively. **Lanes 3 and 4** are kidney DNA from transgenic from non-expressing mice and expressing mice, respectively. The 9.5kb fragment represents a complete digest with XhoI and suggest that the intronic region is not methylated. With respect to tissues isolated from Pgk-1,2-lacZ^{off} mice, expression within the testis and not within the kidney also does not correlate with methylation. (**C**) An ethidium bromide stained gel shows that digestion of DNA is complete with BglII and XhoI under the selected conditions. Plasmid alone (**lane 2**) and Plasmid seeded in genomic DNA (**lane 1**).


A
Probe
Exon 1
2
Lac Z
3
Bgl II
XhoI
Xho I
Bgl II
16kbp
11.5kbp
9.5kbp


B
1
2
3
4
16kb
11.5kb
9.5kb

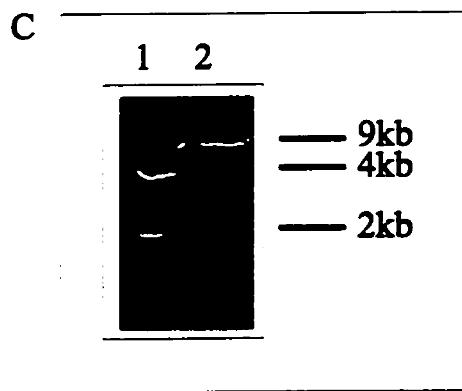
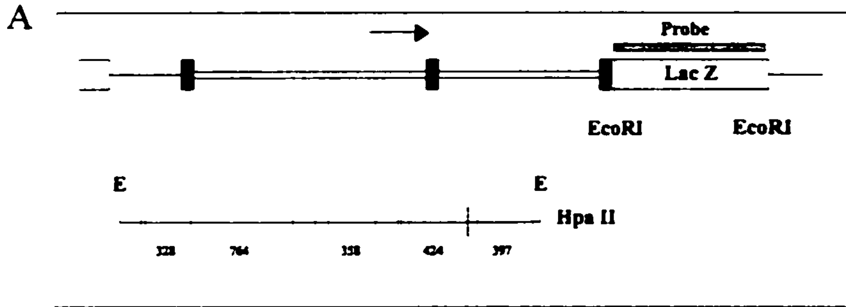
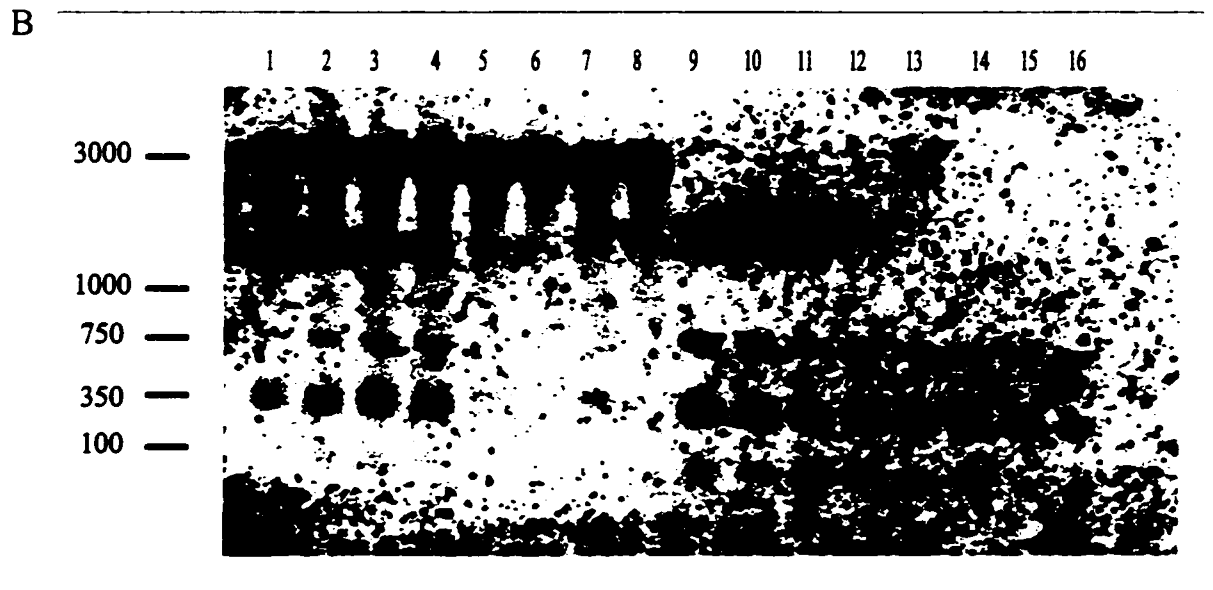

C
1
2
9kb
4kb
2kb

Figure 9: Methylation is associated with the lacZ region of maternally and paternally imprinted Pgk-1,2-lacZ.

DNA was isolated from the kidney and testis of both Pgk-1,2-lacZ^{on} and Pgk-1,2-lacZ^{off} mice. Testicular transgene expression is common to both phenotypes but kidney expression is limited to Pgk-1,2-lacZ^{on} mice. DNA was digested with EcoRI and MspI (5μg DNA- **B lanes 9-16**) or its isoschizomer HpaII (20μg DNA- **B lanes 1-8**) and restriction fragments analyzed by southern blotting. (**A**) A diagram of the Pgk-1,2-lacZ transgene shows restriction sites for MspI, HpaII and EcoRI within the lacZ gene. (**B**)The lacZ region is heavily methylated in all DNAs tested as indicated by the failure of HpaII to digest a 3.0kb EcoRI fragment (**lanes 1-8**). MspI digests of the same DNA resulted in the detection of expected 750bp, 350bp and 100bp fragments (**lanes 9-16**). A small amount of the MspI digestion pattern can be observed for HpaII digests of DNA isolated from transgene expressing tissues suggesting that some transgene copies may be unmethylated (**lanes 1-4,5,**7). HpaII digested kidney DNA from Pgk-1,2-lacZ^{off} mice show no signs of demethylation (**lanes 6,8**). **Lanes 1, 3, 9, and 11** testis DNA from Pgk-1,2-lacZ^{on} mice; **lanes 2, 4, 10, and 12** kidney DNA from Pgk-1,2-lacZ^{on} mice; **lanes 5,** 7, **13, and 15** testis DNA from Pgk-1,2-lacZ^{off} mice; **lanes 6, 8, 14, and 16** kidney DNA from Pgk-1,2-lacZ^{off} mice. (**C**) An ethidium bromide stained gel shows that digestion of DNA is complete with EcoRI and HpaII or MspI under the selected conditions. Plasmid alone digested with MspI(**lane 3**), plasmid seeded in genomic DNA digested with MspI (**lane 1**) and plasmid seeded in genomic DNA digested with HpaII (**lane 2**).


A
Probe
Lac Z
EcoRI
EcoRI
E
E
Hpa II

C
1 2 3


B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
3000
1000
750
350
100

could only be resolved by increasing the amount of DNA loaded by 4X or to 20μg. Heavy methylation of the lacZ gene was present in all DNA samples regardless of whether tissues expressed the transgene or not (Fig 9B lanes 1-8). Only a small number of transgenes appear to be under methylated.

Plasmids often integrate as multicopy concatemers and has been shown to occur in mice and cell culture.(38) It is thus unclear what proportion of transgenes are active in any one cell. Since the Pgk-1 promoter is a very active promoter, all transgene copies are assumed to be silent in non-expressing tissues or cells. In expressing tissues, a large proportion of transgenes are methylated within the lacZ region. A small proportion remains unmethylated. indicated by MspI banding patterns present in HpaII digested DNA (Fig 9B lanes 1-5, 7). Whether or not the demethylated transgenes are responsible for tissue expression remains to be elucidated. It is difficult to quantitate of the number of demethylated transgenes to compare to the numbers of expressing cells since uniform cellular expression is absent from all expressing tissues.

Treatment of Mouse Embryonic Fibroblasts From Maternally Inherited Transgenics With 5-Aza-2-Deoxycytidine Results in Transgene Reactivation

5-Aza-Cytidine or analogues are potent demethylating agents. Use of these demethylating agents have resulted in the reactivation of silent genes [29,65]. To further determine if methylation was involved in inactivation of our transgene, mouse embryonic fibroblasts (MEF) carrying maternally inherited Pgk-1,2-lacZ transgenes were treated with 5-Aza-2-Deoxycytidine (5A2C). MEFs were isolated from day 12-13 embryos carrying one copy of maternally inherited Pgk-1,2-lacZ. Treatment of MEFs with 3.0μm 5A2C for 48hr

or approximately two cell divisions, resulted in reactivation of the transgene in 2.48% of treated cells with a standard deviation of 0.45% (Fig 10A,B). Control cells which were not treated with 5A2C spontaneously reactivated the transgene in $< 1*10^{-6}$ cells. This data would suggest that methylation is responsible for transgene repression.

De Novo Methylation of the LacZ Gene During Embryogenesis

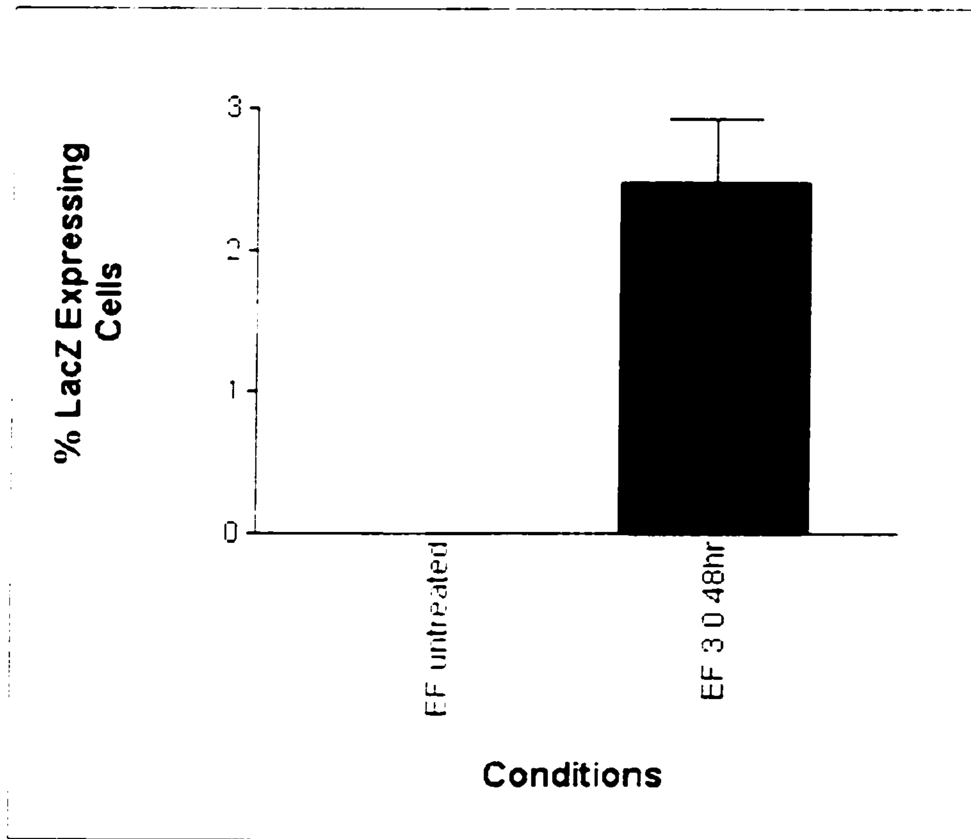
Was methylation within the lacZ region responsible for this phenomena and is it established during gametogenesis or embryogenesis? New Pgk-1,2-lacZ transgenics were created to address these questions. Additionally some of the new transgenic lines were also created within FVB/N backgrounds instead of C3H/C57BL to determine any influence of genetics on imprinting and/ or methylation. C57BL and BALB/c genetic backgrounds have often been associated with de novo transgene methylation whereas FVB/N and DBA2 backgrounds have been linked to transgene hypomethylation.[12]

Transgenes in founder animals can only be developmentally modified and thus provide an excellent opportunity to investigate whether de novo methylation of the Pgk-1,2-lacZ transgene occurred during gametogenesis or embryogenesis. Kidney DNA was isolated from founder animals and digested with EcoRI and MspI or HpaII. Subsequent hybridization with a lacZ probe revealed that DNA from all founder animals tested were also subject to methylation (Fig 11). Thus methylation within the lacZ region of the transgene is established during embryogenesis. Slight demethylation of the lacZ region is again present only in kidneys which strongly express the transgene, as indicated by the presence of MspI profiles (Fig 11B lanes 1, 6, 9, 11, 13-16, 18). Whether demethylation of a small proportion of transgenes is responsible for activity remains to be elucidated.

Figure 10: Treatment of MEFs from Pgk-1,2-lacZ^{off} mice with 5A2C reactivates transgene expression.

Mouse embryonic fibroblasts were isolated from day 12-13 embryos carrying maternally transmitted transgenes, and were subjected to no treatment or 3.0μm 5A2C for 48hr. Subsequent fixation with 0.02% glutaraldehyde and staining with X-gal resulted in 2.48% positive cells, with a standard deviation of 0.45% (**A, B panel 2**). Untreated cells expressed the transgene in $<1*10^{-6}$ cells (**A, B panel 1**). (n = 3; 1000 cells counted per trial)

A



B

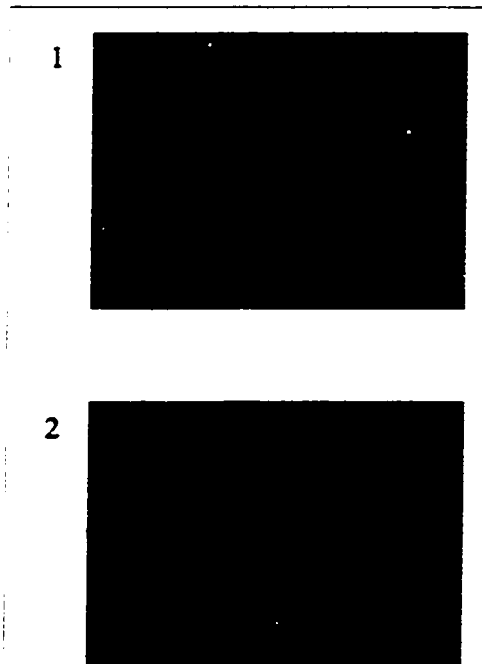
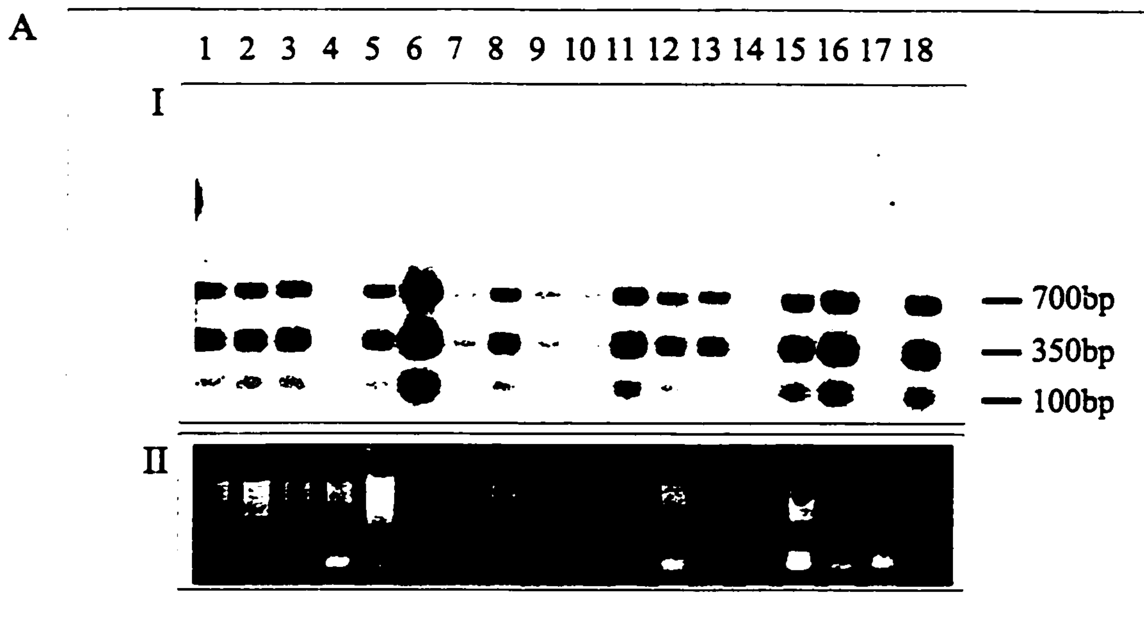
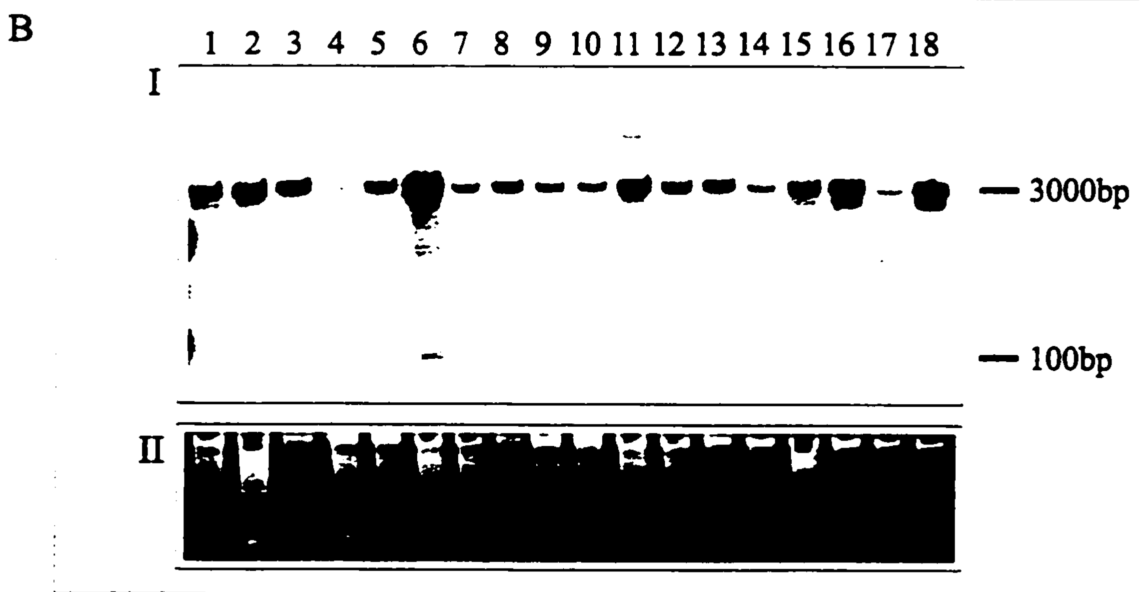


Figure 11: HpaII sites of the LacZ gene are methylated during embryogenesis.

DNA was isolated from the kidney of 18 adult Pgk-1,2-lacZ founders and 10µg of DNA was digested either with EcoRI and MspI or its methylation sensistive isoschizomer HpaII. The restriction fragments were analyzed by southern analysis using a 2.3kb lacZ probe. Autoradiography shows that digestion of kidney DNA with EcoRI and MspI resulted in complete digestion of the the lacZ gene yielding the characteristic 700, 350 and 100bp bands (**A panel I**). Digestion with EcoRI and HpaII revealed that the lacZ gene was heavily methylated, indicated by the presence of a predominant 3.0kb undigested fragment (**B panel II**). Smaller restriction fragments are present in **B lanes 1, 6, 8, 11, 13-16, 18** which indicate that some transgene copies are not methylated. DNA samples in **B lanes 1, 6, 8, 11, 13-16, 18** were isolated from strongly expressing tissues and thus may correlate with slight demethylation within the lacZ region. Two samples in **B lanes 4 and 17** are have respectable tissue expression expression but do not show any demethylation. **A panel II** and **B panel II** are accompanying ethidium bromide stained gels visualized under UV illumination. Transgenic mice are denoted as follows: M1 (**1**), M2 (**2**), M3 (**3**), M4 (**4**), F1 (**5**), F2 (**6**), F3 (**7**), F4 (**8**), 2936 (**9**), 2931 (**10**), 2946 (**11**), 3010 (**12**), 2942 (**13**), 2923 (**14**), 2914 (**15**), 3125 (**16**), 2921 (**17**), and 2929 (**18**).


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
I
700bp
350bp
100bp
II


B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
I
3000bp
100bp
II

No difference with respect to methylation or imprinting was detected in mice with either FVB/N or C3H/C57BL backgrounds. Genetic modifiers within these two strains appear to have little influence on the epigenetic modification of the Pgk-1,2-lacZ transgene.

Variable Tissue and Cellular Expression of the Pgk-1,2-LacZ Transgene

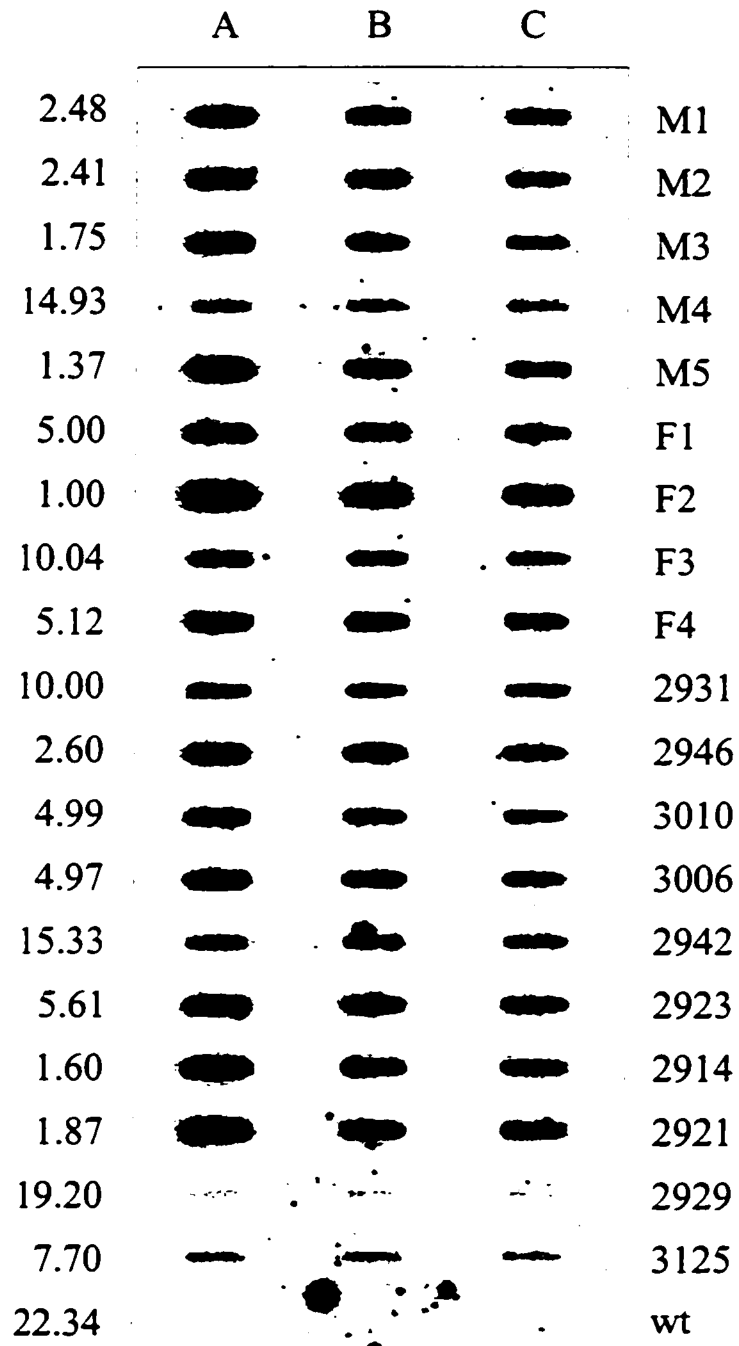
Histological analysis of various tissues from the new transgenic lines revealed that expression patterns and levels were different from line to line. Genetic modifiers in mice with FVB/N and C3H/C57BL backgrounds did not appear to influence expressivity of the transgene and expression pattern. Mice with either backgrounds showed great variability in their tissue and cellular expression and in the levels of expression as well. Table I is a summary of expression and the level of expression from various tissues of 19 founder animals.

DNA was isolated from founders animals to determine copy number and its relation to expression. Generally expression is correlated with low copy number.(44) Slot blot analysis (Fig. 12) failed to reveal any good correlation between expression and copy number. Some mice had high copy numbers with good expression in most tissues (Kidney- Table 1. Fig 13A) while others had low expression(Kidney- Table 1, Fig 13B). Similarly for mice with low copy numbers(Table 1. Fig13C, D, E).

Although a direct DNA analysis was not performed, variability in expression levels and patterns suggests that the Pgk-1,2-lacZ transgene is prone to position effects. Expression of many transgenes are influenced regulatory DNA elements close to the position of integration.(26.43) The Pgk-1,2-lacZ transgene appears to be no exception.

Fig 12: DNA copy number determined by slot blot analysis

DNA was isolated from the kidney of 19 adult Pgk-1,2-lacZ founders. Copy number was analyzed by slot blot analysis. DNA was blotted onto a nylon membrane, and hybridized to a 2.3kb lacZ probe. The amount of blotted DNA is indicated for **collumn A**. **Collumn B and C** have 20% and 10%, respectively, of the amount of DNA in **collumn A**. The transgenic founders from which DNA samples were isolated are indicated for each row. The last row is kidney wild-type negative control. Quantitations were determined using Molecular Dynamics Phosphorimager SI.



A
B
C
2.48
M1
2.41
M2
1.75
M3
14.93
M4
1.37
M5
5.00
F1
1.00
F2
10.04
F3
5.12
F4
10.00
2931
2.60
2946
4.99
3010
4.97
3006
15.33
2942
5.61
2923
1.60
2914
1.87
2921
19.20
2929
7.70
3125
22.34
wt

Figure 13: Tissue expression in the kidney of transgenic founders

Kidneys from transgenic founders were frozen and 12μm sections cut. Sections were X-gal stained and counterstained with nuclear fast red, before mounting. There is variable expression in terms of level of expression and mosaicism within all expressing tissues. Representative kidney sections are shown. Founder M2 (**A**); 2946 (**B**); 2929 (**C**); 3125 (**D**); and 2931 (**E**). The bar in **A** is 10μm.

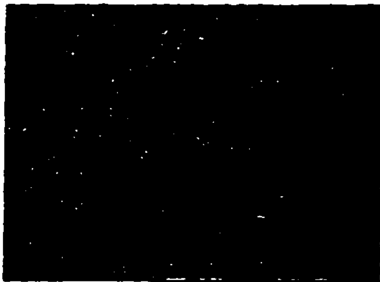
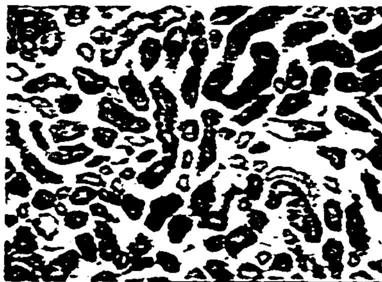
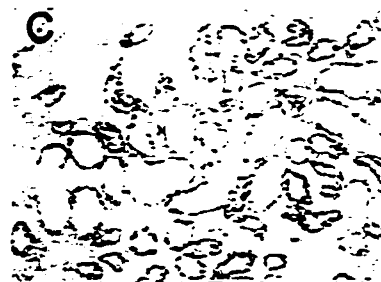
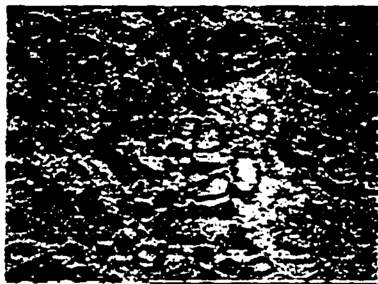
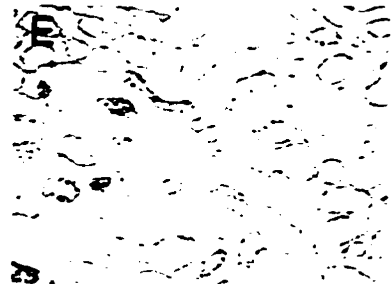






Table 1: Summary of hostological analysis of lacZ expression in various tissues from Pgk-1,2-lacZ transgenic founders

Founder #	Tissue	Staining	Density	Intensity	Copy #	Strain	Sex
F1	Brain	y	++	+++	63	C3H/C57	F
	Heart	y	+	++			
	Kidney	y	+	++			
	Liver	n	-	-			
	Calf Muscle	n	-	-			
	Spleen	n	-	-			
F2	Brain	y	+	+++	728	C3H/C57	F
	Heart	y	+	++++			
	Kidney	y	++	+++			
	Liver	n	-	-			
	Calf Muscle	n	-	-			
	Spleen	y	+	++			
F3	Brain	y	+++	+++	16	C3H/C57	F
	Heart	y	+	++			
	Kidney	y	++	+++			
	Liver	n	-	-			
	Calf Muscle	y	+	++			
	Spleen	y	+	+			
F4	Brain	y	++	+++	73	C3H/C57	F
	Heart	y	+++	+++			
	Kidney	y	+++	+++			
	Liver	y	+	++			
	Calf Muscle	y	+++	++			
	Spleen	y	+	++			
2946	Brain	y	++++	++++	121	FVB/N	F
	Heart	y	+++	+++			
	Kidney	y	+++	+++			
	Liver	y	+++	+++			
	Calf Muscle	y	+++	+++			
	Spleen	y	+	+			
2931	Brain	y	+	+++	16	FVB/N	F
	Heart	n	-	-			
	Kidney	y	++	+			
	Liver	n	-	-			
	Calf Muscle	n	-	-			
	Spleen	y	+	+			
2914	Brain	y	+	++++	224	FVB/N	F
	Heart	y	+	+++			
	Kidney	y	+++	+++			
	Liver	y	+	+			
	Calf Muscle	n	-	-			
	Spleen	n	-	-			
2929	Brain	y	+	++++	1	FVB/N	F
	Heart	n	-	-			
	Kidney	y	+++	+++			
	Liver	n	-	-			
	Calf Muscle	n	-	-			
	Spleen	n	-	-			

Founder #	Tissue	Staining	Density	Intensity	Copy #	Strain	Sex
3125	Brain	y	++	+++	6	FVB/N	F
	Heart	y	+	+++			
	Kidney	y	+++	+++			
	Liver	y	++	+++			
	Calf Muscle	n	-	-			
	Spleen	y	+	++			
2923	Brain	y	+	++	67	FVB/N	F
	Heart	y	+	+++			
	Kidney	y	+++	+++			
	Liver	y	++	+++			
	Calf Muscle	n	-	-			
	Spleen	y	+	+			
2942	Brain	y	+++	+++	12	FVB/N	F
	Heart	y	+++	+++			
	Kidney	y	++	++			
	Liver	n	-	-			
	Calf Muscle	n	-	-			
	Spleen	y	++	++			
3006	Brain	y	++++	++++	57	FVB/N	F
	Heart	y	+++	+++			
	Kidney	y	+++	+++			
	Liver	y	+++	+++			
	Calf Muscle	y	+++	+++			
	Spleen	y	+	+			
2921	Brain	y	++	+++	223	FVB/N	F
	Heart	y	++	++			
	Kidney	y	+++	+++			
	Liver	y	++	+++			
	Calf Muscle	n	-	-			
	Spleen	n	-	-			
M1	Brain	y	+++	++++	107	C3H/C57	M
	Heart	y	++	+++			
	Kidney	y	+++	+			
	Liver	y	++	+			
	Calf Muscle	y	++	++			
	Spleen	n	-	-			
	Testis	y	++	++++			
M2	Brain	y	+	+++	94	C3H/C57	M
	Heart	n	-	-			
	Kidney	n	-	-			
	Liver	n	-	-			
	Calf Muscle	n	-	-			
	Spleen	y	+	++			
	Testis	n	-	-			

Founder #	Tissue	Staining	Density	Intensity	Copy #	Strain	Sex
M3	Brain	n	-	-	120	C3H/C57	M
	Heart	n	-	-			
	Kidney	n	-	-			
	Liver	n	-	-			
	Calf Muscle	n	-	-			
	Spleen	y	+	+			
	Testis	n	-	-			
M4	Brain	y	++	+++	5	C3H/C57	M
	Heart	y	++	++			
	Kidney	y	+++	+++			
	Liver	y	+	+			
	Calf Muscle	y	++	++			
	Spleen	y	+	++			
	Testis	y	++	+++			
M5	Brain	y	+	++	263	C3H/C57	M
	Heart	n	-	-			
	Kidney	y	+++	+			
	Liver	n	-	-			
	Calf Muscle	y	+	++			
	Spleen	y	++	++			
	Testis	y	+++	+++			
3010	Brain	y	+	+++	42	FVB/N	M
	Heart	y	+++	+++			
	Kidney	y	+	++			
	Liver	y	++	+++			
	Calf Muscle	n	-	-			
	Spleen	y	++	++			
	Testis	y	+	+++			

Discussion and Conclusions

Expression of the Pgk-1,2-lacZ transgene in one of our transgenic lines is subject to unusual parental inheritance patterns. Paternal inheritance of the transgene results in widespread expression of lacZ, similar to previously published results. [69] Maternal transmission of the transgene results in inactivation in most tissues with the exception of brain, testis and heart. Additionally, modification of Pgk-1,2-lacZ by maternal inheritance renders the imprint irreversible, despite retransmission through the male germline.

Transgene imprinting effects have been previously described [3,13,20,33,92,101,106] and suggested to represent a common phenomenon in transgenic mice [106]. Endogenously imprinted genes and imprinted transgenes share many features. These include parent specific expression patterns, reversibility of transgenic imprints and a correlation between hypermethylation and inactivation. [13,85,91,101,106] Only two examples have been reported in which transgene imprints do not reflect characteristics of natural imprinting phenomena. The first is a cumulative epigenetic modification of a transgene passaged through the female germline that is rendered irreversible after the third generation. [3] The second is the irreversible inactivation of a HBV transgene when maternally inherited. [33]

Unlike, most parent of origin effects that have resulted in complete inactivation of the transgene [3,20,33,101], maternally transmitted Pgk-1,2-lacZ is expressed within the brain (ependyma and caudate putamen), testis and heart. The IGF-2 gene is inactivated after maternal inheritance but is ectopically expressed within a few adult tissues. [19]

Extensive examination of lacZ expression of maternally inherited Pgk-1,2-lacZ in the brain revealed an unappreciated expression pattern within the ependyma lining the

ventricular system. Expression is manifested in a symmetrical striped pattern, particularly evident within the third ventricle and aquaduct, and does not seem to be limited by ependymal cell type. Two possible explanations may be possible. LacZ expression represents a yet uncharacterized lineage of ependymal cells or that transgene expression is influenced by the regional position of ependymal cells. A more detailed examination is necessary to determine the nature of expression within these cells.

DNA methylation is correlated with gene inactivation and has been suggested to be the mechanism of epigenetic modification in genomic imprinting and X Chromosome inactivation. Imprinted transgenes have also been associated with increased methylation. [13,85,91,101,106] We analyzed two regions within paternally and maternally inherited Pgk-1.2-lacZ transgenes: intron 1 and lacZ.

Methylation of XhoI sites in intron 1 has been associated with the inactivation of Pgk-1 on the inactive X chromosome.[5] DNA was isolated from kidney and testis to represent expressing and non expressing tissues. The lacZ transgene is expressed in the testis regardless of transmitting parent, whereas kidney expression is restricted to paternally inherited transgenes. We found that methylation was absent from two XhoI sites in intron 1 of testis and kidney DNA of maternally and paternally transmitted transgenes.

Inactivation of a K18-lacZ fusion gene was correlated with methylation within the lacZ region. [106] We sought to investigate whether a similar situation existed for the Pgk-1.2-lacZ transgene. The lacZ region was found to be highly methylated in the kidney and testis regardless of parental inheritance or expression. Under methylation of a small number of transgenes was only evident in expressing tissues. This suggested to us that lacZ expression is driven from a small number of unmethylated transgenes and that remaining

methylated transgenes are inactive. Since, microinjection of DNA usually results in multicopy concatemers, it is difficult to assess what proportion of transgenes are active in any one cell. Lack of transgene expression would mean that all copies are silent. In *Drosophila* and yeast, inactivation spreads from a nucleation center, and is dependent of concentrations of repressive and activating factors [4]. Derepression of genes seemingly occurs at the boundary of repressive complexes. Perhaps, a similar situation exists in transgene arrays in mice.

Prokaryotic sequences especially lacZ, are responsible for improper regulation of various transgenes. The HMG, CMV, K18, and H-2Kb promoters drive widespread expression of many transgenes.[60,76,93,106] Fusion of these promoters with lacZ abolishes regular expression patterns.[15,50,78,112] Furthermore inclusion of lacZ disrupts the insulatory effects of SARs and LCRs. [18,32] Prokaryotic sequences have a higher amount of CpG sequences than most eukaryotic DNA. Perhaps this feature renders them susceptible to DNA modification or targets them as cis-acting elements. A methylated triplet CpG sequence is responsible for nucleosome positioning in vitro.[17] Nucleosomes can control the accessibility of transcription factors to their DNA targets. Methylation of the triplet CpG excludes nucleosomes similar to AT rich regions in SARs. The lacZ gene contains two such sequences. It is possible that the lacZ gene may act as a boundary for nucleosome formation and enforce abnormal nucleosome positioning. The severity of such remodeling probably depends on other factors which modulate positioning. The notion that methylation exerts its effects on gene regulation through nucleosome positioning is attractive. Time dependence of repression from methylated templates [47] and reactivation through 5A2C [39] are consistent with chromatin remodeling.

To further determine the influence of methylation on the Pgk-1,2-lacZ transgene we isolated hemizygous MEFs carrying maternally inherited transgenes and treated them with 5A2C. 5AC and analogues are demethylating agents and act by covalently binding DNA methyltransferase. Treatment of MEFs with 5A2C resulted in transgene reactivation in a 2.48% treated cells with a standard deviation of 0.45%. Untreated cells express the transgene in < $1*10^{-6}$ cells. Conditions for 5A2C treatment were not optimized and may have contributed to a low reactivation percentage. Nevertheless the success of transgene reactivation using a demethylating agent suggests that methylation may be responsible for inactivation of the transgene.

Additional transgenic founders were created to determine whether unusual transgene effects in the original line could be duplicated. Transgenic mice were created in two strain backgrounds; FVB/N and C3H/C57BL, to determine effects of genetic strain modifiers. DBA2 and FVB/N genetic backgrounds have been associated with transgene hypomethylation, whereas BALB/C and C57BL strains have been correlated with transgene hypermethylation. [12] Initial breeding of the original strain into a CD1 background had no discernible effects on transgene expression. Mating experiments are still underway, to determine if parent of origin effects are also associated with the new lines.

We proceeded to characterize methylation and transgene expression within 19 of the founder animals. Methylation was present within the lacZ region of kidney DNA from the 19 founders. This suggested that transgene methylation did not require gametogenesis and occurred during embryogenesis. Similar to the original strain, slight hypomethylation was detected in kidneys which strongly expressed the transgene. Only 2 of 11 founders strongly expressed the transgene within the kidney but were not associated with hypomethylation.

Histological analysis of various founder tissues revealed expression patterns and levels that varied between all the founders. Mosaicism or heterocellular expression was a common feature within all expressing tissues. Although direct analysis was not done, our results suggest that the Pgk-1,2-lacZ transgene is subject to position effects.

An examination of copy number through slot blot analysis revealed no correlation with expression. Generally expression is correlated with low copy number. [22] There were founders with high and low copy numbers which expressed or did not express the transgene in various tissues.

In conclusion, an unusual Pgk-1,2-lacZ transgenic line has been found to be associated with imprinting. Paternal inheritance results in widespread expression patterns whereas maternal inherited transgenes are irreversibly inactivated in a variety of tissues. The lacZ region of our transgene is heavily methylated regardless of parental transmission or expression. Slight under methylation of the lacZ gene, characteristic of only expressing tissues, and reactivation of maternally inherited transgenes by 5A2C, suggests that methylation may be related to inactivation of Pgk-1,2-lacZ. Similar methylation analysis of the lacZ region in 19 Pgk-1,2-lacZ founders reveal that modification of lacZ is established during embryogenesis. There is extensive variability in expression patterns and level of expression in tissues from the 19 founders suggesting that the Pgk-1,2-lacZ is prone to position effect. However, we have not yet established the cause of imprinting within the original strain. It is unlikely that parental effects are due to site of integration since phenotypic abnormalities were not detected on hemizygous or homozygous backgrounds.

Chapter 2

Abstract

We sought to determine if homogeneous transgene expression could be maintained in ES cells and mice, harbouring a singly intergrated transgene. Homologous recombination of a promoterless ires-(geo cassette into the Clk/ Sty gene resulted in unusual transgene effects in ES cells and in mice. The promoterless cassette allowed expression to be driven from an unaltered ubiquitous endogenous promoter and IRES allows translational initiation from internal sequences. (Clk/ Sty knockout ES cells and mice were a kind gift from Dr. John Bell's lab)

Sty/ Clk hemizygous ES cells were found to express the transgene in a mosaic fashion and subcloning revealed that heterogeneity was a result of gene loss. Transgenic mice were derived from the same ES cells. Histological analysis revealed that expression of the transgene was limited to the heart and testis, despite ubiquitous expression of endogenous Sty. Northern and southern analysis suggests that the nature of heterogeneous expression in these transgenics is gene inactivation.

Our results suggest that the problems associated with transgenes are more complicated than previously thought. Controlling copy number, site of integration and cis-elements was not able to alleviate unusual transgene effects. Furthermore, the nature of heterogeneous transgene expression appears to be mechanistically different in ES cells and in animals.

Clk/ Sty interacts with members of the serine/ arginine-rich (SR) family of splicing factors.[16,24]

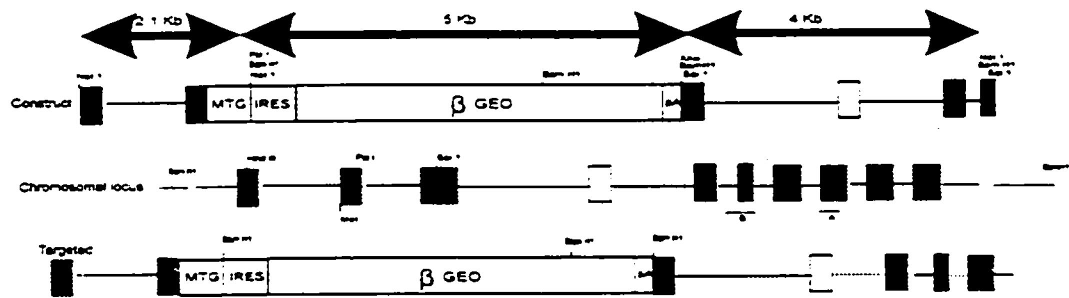
Our colleagues sought to determine the biological significance of Clk/ Sty by targeted disruption of the Clk/ Sty gene in mice. Homologous recombination of a promoterless internal ribosome entry segment (ires)-(geo cassette into exon 2 resulted in a truncated non-functional Clk/ Sty protein in ES cells (Fig 14-This figure was constructed by Jeniffer Ingram). These ES were subsequently used to generate transgenic mice. Unfortunately no discernible phenotype has been detected in homozygous KO mice.

We report here that (geo expression in ES cells with ires-(geo disrupted Clk/ Sty. is mosaic and that the nature of expression is due to gene loss. Homozygous transgenic mice derived from the same ES cells result in a loss of (geo expression in all tissues tested except for testis and heart. In this case. it is gene inactivation and not gene loss that is responsible for the silencing of the Sty promoter.

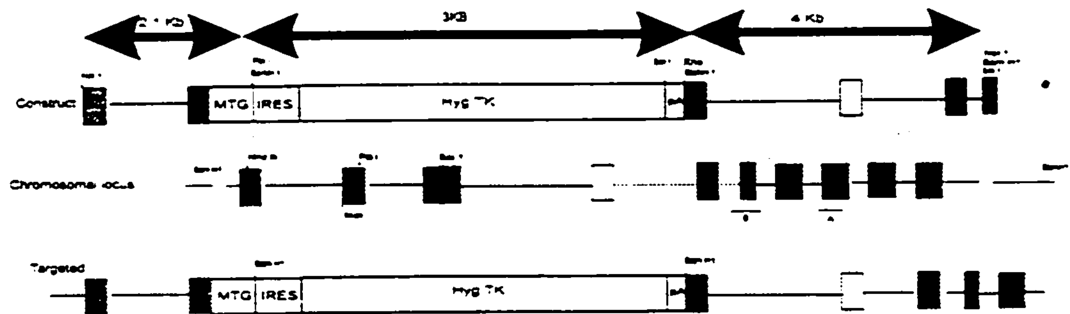
Figure 14: Sty DNA targeting constructs

These constructs were used to target the Sty gene and create a truncated in active protein. Sty-IRES-β-geo (**a**) was used to create hemizygous Sty KO ES cells. Sty-IRES-HygTK (**b**) was used to target the remaining allele in hemizygous Sty KO eS cells. Dark boxes represent exons and the clear box represents Sty's alternatively spliced exon. pA is the simian virus polyA signal (SV40); MTG is a myc tag; β-geo is a fusion gene between lacZ and neomycin; HygTK is a fusion gene between hygromycin and thymidylate synthase; and IRES is an internal ribosomal entry site.

a) *sty*-IRES-β *geo*



b) *sty*-IRES-*Hyg TK*



Materials and Methods

Cell Culture

Embryonic Stem (ES) cells were cultured in minimum essential medium alpha (MEM() (Gibco) containing 1% leukemia inhibitory factor (LIF) from CHO cell conditioned media and $3*10^{-6}$% (-mercaptoethanol supplemented with 15% fetal bovine serum (Cansera International Inc). The cells were maintained in plastic tissue culture dishes, which were placed at 37°C in a 10.0% CO2 incubator. They were kept in undifferentiated state by passaging cells before sub-confluency. Briefly, the cell monolayer was washed in phosphate-buffered saline (PBS) (0.8% NaCl, 0.2% KCl, 0.02% KH_2 PO_4 and Na_2HPO_4), and then incubated in trypsin-EDTA (1mM EDTA and 0.025% trypsin in PBS) for 10 minutes. The detached cells were dispersed by vigorous pipetting. They were then counted and replated at a density of 200cells/mm2 into fresh medium.

Clones of ES cells were isolated by plating cells at low density onto monolayers of irradiated MEF or STO cells. Colonies were picked and expanded to isolate DNA and RNA. Clones were also fixed in 0.2% glutaraldehyde in PBS for 1 hour, washed and incubated overnight at 37°C in X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside).

Histological Analysis

Mouse tissues were prepared from homozygous and wild type animals that were anesthetized and perfused through the heart with PBS followed by Lanas fixative (4% paraformaldehyde and 0.2% saturated picric acid in 0.16M sodium phosphate buffer at pH 6.9). Tissues were removed, postfixed for 90 minutes and transferred to 10% sucrose in 100mM phosphate buffer. Tissues were frozen and 12μm cryostat sections were cut, thaw

mounted onto uncoated slides (Superfrost Plus, Fisher Scientific), stained with X-gal, and counterstained with either nuciear fast red for 1-2 minutes before mounting.

Probes

Plasmid DNA fragments which were used as probes were isolated as follows. Briefly, plasmid DNA was digested using appropriate restriction endonucleases under the conditions recommended by manufacturer. DNA was separated by electrophoresis through 1% low melt agarose gels in 0.1X TE. The appropriate DNA fragments were isolated by through cuts into the agarose gels which were recovered by dissolving gel fragments in TE at 55oC, extracted with phenol-chloroform (1:1), and finally redissolved in TE. The LacZ probe was a 2.3kb EcoRI-ClaI fragment from pDM2, the (-tubulin probe was a full length mouse cDNA and the hygromycin probe was a 1.0kb BamH1 fragment from PKJ23. Probe DNAs were 32P-dCTP labeled using the standard multiprime DNA labelling protocol (Amersham).

DNA Isolation and Electrophoresis

Genomic DNA was extracted from mouse Embryonic Stem (ES) cells and tissues as previously described [51]. PBS washed cells and finely minced tissues were lysed in DNA lysis buffer (0.2% SDS, 200mM NaCl, 5mM EDTA, and 100mM Tris HCl (pH 8.5) containing 100(g/mL proteinase K). After incubation at 37°C for 2 hours and 15-24hr with gentle agitation, respectively, one volume of isopropanol was added to recover DNA from the lysate. The DNA was dissolved in TE (10mM Tris HCl (pH 8) and 1mM EDTA).

Purified genomic DNA was digested by restriction endonucleases at a concentration of 10U/ μg. DNA fragments were separated by electrophoresis through 1.0% agarose gels in

0.1X TE. Separated DNAs were transferred to Hybond-N membranes (Amersham) and treated with ultraviolet light at 110mJ using a GS Gene linker UV chamber (Biorad).

Northern Blot

RNA was isolated using TRIzol (Gibco BRL). Briefly, monolayer cells or tissue homogenates were lysed by addition of TRIzol. Following incubation at room temperature for 5 minutes 0.2mL chloroform/ mL TRIzol was added to the sample and incubated for an additional 2 minutes. Samples were centrifuged at 12,000g for 15 minutes at 4°C and aqueous phase mixed with 0.5mL isopropanol/ mL TRIzol. Following a 10 minute incubation at room temperature, samples were centrifuged at 12,000g for 10 minutes at 4°C. After washing with 70% ethanol, the RNA was dissolved into Rnase-free water.

RNA was separated by electrophoresis through 1% agarose gels in 20mM 3-[N-morpholino]propane-sulfonic acid, 1mM EDTA, 5mM sodium acetate at pH 7, and 10% formaldehyde. Separated RNAs were transferred to Hybond-N membranes (Amersham) and treated with ultraviolet light at 110mJ using a GS Gene linker UV chamber (Biorad).

Probe Hybridization

Prehybridization was carried out in 50% deionized formamide, 5 x SSPE, 2.5 x Denhardt's solution, 0.1% SDS, 0.2mg/mL denatured salmon testis DNA (1 x SSPE is 0.15M NaCl, 10mM $Na_2H_2PO_4$, and 1mM EDTA). Membranes were hybridized for 15-24 hours at 42°C in prehybridization solution containing 32P-dCTP labeled probes. Following incubation, membranes were washed once in 2 x SSC and 0.1% SDS at 42°C for 15 minutes, followed by several changes of 0.2 x SSC and 0.1% SDS at 65°C for 45 minutes. Radioactivity was visualized and analyzed using Molecular Dynamics Phosphorimager SI.

Results

Single Copy Integrations are subject to mosaic expression

One allele of Clk/ Sty was disrupted in ES cells by homologous recombination of a promoterless Ires-(geo sequence. The advantage of using a promoterless construct is the ability to determine the proper regulation of endogenous promoters. The Ires DNA element allows (geo to be translationally initiated from internal sequences. βgeo is a fusion between E. Coli lacZ and neomycin, allowing easy visual detection of positive cells, and selection capability, respectively.

We were provided with a neomycin selected ES cell clone (21A1) carrying one normal copy of Clk/ Sty and one βgeo disrupted allele, by our colleagues. These cells were fixed ad stained with X-gal to determine whether cells expressed (geo uniformly. We found that clone 21A1 expressed (geo in a mosaic fashion (Fig 15A). Despite an attempt to control factors responsible for transgene effects ES cells still expressed the transgene in a mosaic fashion. It is possible that an essential intronic regulatory element was disrupted which altered proper regulation of the Clk/ Sty promoter. If this were true, we would expect that abnormal regulation would be recapitulated in all cells. This is not the case.

The nature of heterocellular expression is the result of gene loss

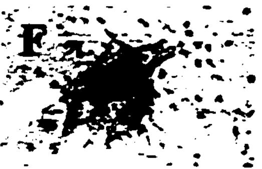
To determine the nature of mosaicism in clone 21A1, cells were plated at low density to select clonal populations. 21A1 ES clones were selected, expanded and harvested for RNA and DNA. Fixation and X-gal staining of ES cell clones revealed that only a few of the clones expressed lacZ and that expression was mosaic (Fig 15F, G). Majority of the clones failed to stain with X-gal (Fig 15B-E). Lack of expression may have been the result of gene

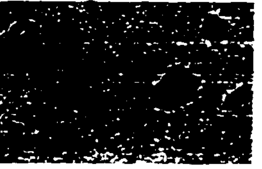
Figure 15: Loss of lacZ expression in Sty hemizygous KO ES cells

Sty hemizygous KO ES cells were fixed and stained with X-gal. Parental cells (**A**) show a mosaic expression of β-geo expression. Parental cell clones were isolated which either expressed (**panels F,G**) or did not express lacZ (**panels B-E**). Expressing clones were also mosaic for expression. The bar in **panel A** represents 10μm.

B

D


F



inactivation, mutations, or gene loss.

Northern analysis of RNA isolated from the parental clones and subclones yielded a 5.3kb transcript coincident with expressing cells. Northern blots were hybridized to a 2.3kb lacZ fragment. The transcript was present in parental cells (Fig 16B lane 8), and two subclones (Fig 16B lane 1,2). Neither gene product nor transcripts were detected for other subclones (Fig 16B lanes 3-7).

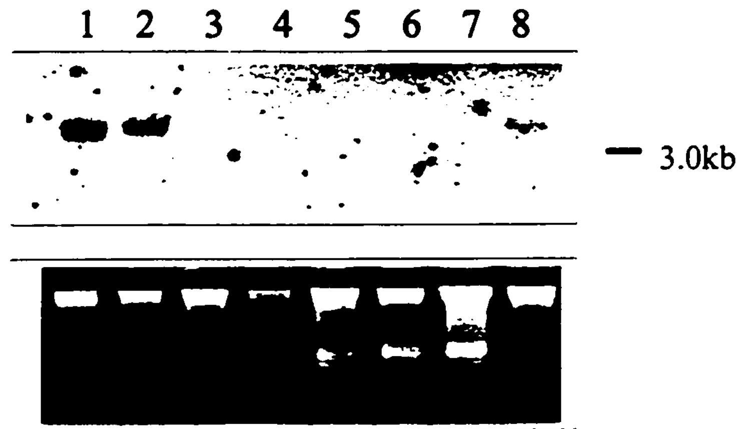
Southern analysis of DNA from the parental clones and subclones were analyzed for presence or absence of (geo sequences to confirm or dismiss the possibility of gene loss. DNAs were digested with BamH1, separated by gel electrophoresis, blotted and hybridized to a 2.3kb lacZ probe. The presence of the ires-(geo DNA is expected to yield a fragment of 3.7kb. Analysis of the DNA revealed that lacZ sequences were absent from subclones which failed to express the transgene (Fig 16A lanes 3-7) and present at the appropriate size in parental clones and two expressing subclones (Fig 16A lanes 1, 2, 8). Presence of a 3.7kb DNA fragment is consistent with detection of RNA transcripts and gene product. Thus gene loss appears to be the nature of lack of expression in the parental clone 21A1. Unfortunately there exists the possibility that the original parental clone was contaminated with wild type ES cells. Parental cells were originally selected in neomycin and subsequently used to generate transgenic mice. To absolutely confirm results, single cells clones would have to be selected, tested for expression, and reselected to isolate subclones. This would provide a direct correlation between lack of expression and gene loss.

Interestingly homozygous disrupted Clk/ Sty ES cell clone (B7) carrying ires-(geo on one allele and ires-hygTK on the other allele, was able to maintain stable and uniform expression in the absence of selection. Single cell subclones of the parent double knockout

Figure 16: Southern and Northern analysis of parental Sty hemizygous knockout ES cells and subclones correlates gene loss with loss of expression

RNA and DNA were isolated from Sty hemizygous knockout ES cells and various sub-clones. These nucleic acids were separated by electrophoresis, blotted and hybridized with with a probe for E. Coli LacZ (**A,B**) and tubulin (**B**). (**A**) Southern analysis of parental Sty KO ES cells (**lane 8**) and various subclones (**lanes 1-7**) shows that the β-geo gene is absent within some clones (**lanes 3-7**) and may have been lost through some gene conversion mechanism. Genomic DNA is present in all lanes as confirmed by the corresponding ethidium bromide stained gel (**A panel II**). (**B**) Northern analysis of lacZ expression in parental cells and subclones reveals that expression is restricted to the parental cells (**lane 8**) and two clones (**lanes 1,2**). A Tubulin probe confirms the presence of intact RNA in all lanes (**B panel II**).

A



B

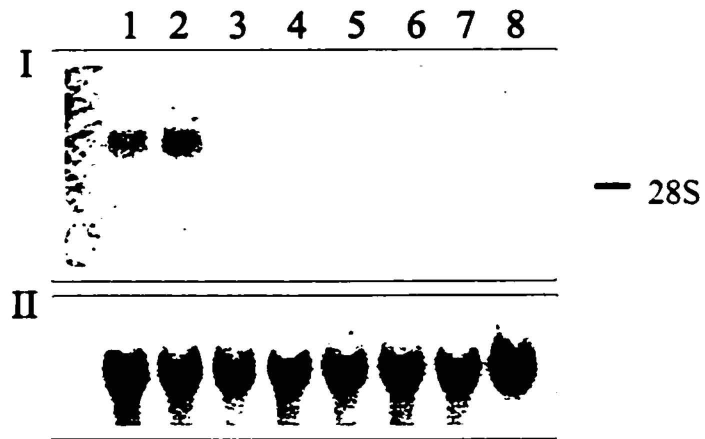
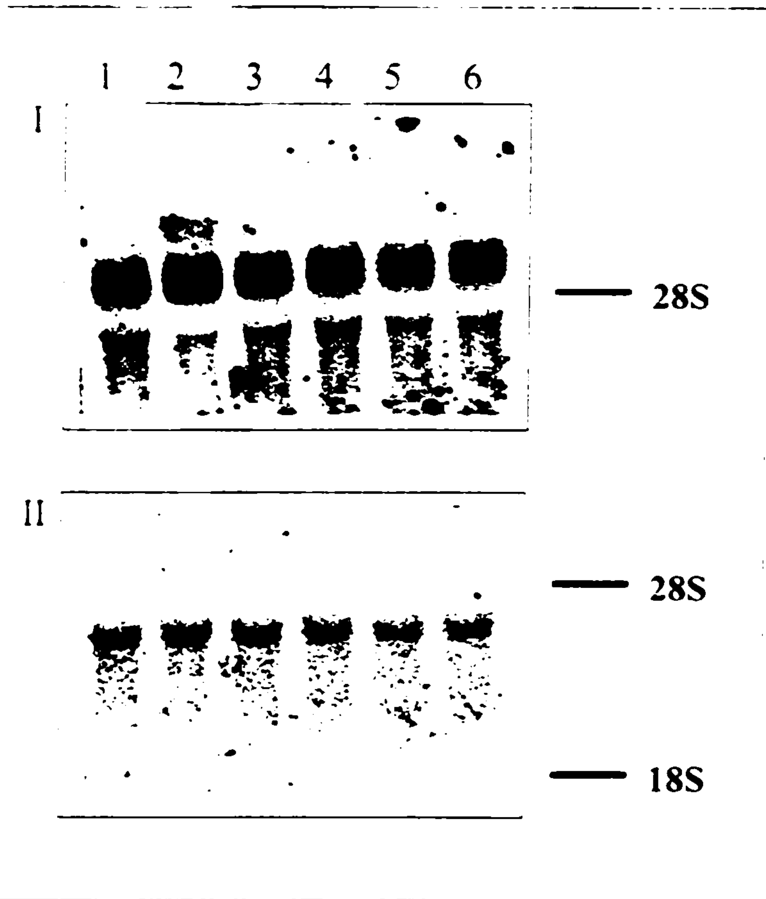


Figure 17: Northern analysis of Sty double KO cells reveals that all subclones express both β-geo and HygTK transcripts, and X-gal staining shows uniform expression

(**A**) RNA was extracted from Sty Double KO cells, subjected to electrophoresis, blotted and probed with lacZ (**panel I**) and hygromycin (**panel II**) probes. Appropriate sized transcripts for lacZ (**panel I**) and hygTK (**panel II**) were present in all subclones. (**B**) X-gal stained Sty double KO ES cells revealed uniform expression of lacZ in all cells. The bar in **B** represents 10μm.


A
1
2
3
4
5
6
I
28S
II
28S
18S

B

ES cell clone also expressed (geo in a uniform fashion (Fig 17B). Northern analysis of RNA isolated from subclones showed transcripts of proper size when probed with a 2.3kb lacZ (Fig 17A panel I) and a 1.0kb hygromycin probe (Fig 17A panel II). Southern analysis of BamH1 digested DNA from duplicate samples yielded fragments corresponding to (geo (Fig 18A) and hygTK(Fig 18B) when hybridized to appropriate probes. Gene loss in hemizygous ES cells may arise through gene conversion as pairing of homologous Clk/ Sty sequences may cause unwanted DNA structures, that are recognized and eliminated by some mechanism. Disruption of both Clk/ Sty alleles may render them unrecognizable by mediators of gene conversion.

The Clk/ Sty promoter is inactive in Homozygous Clk/ Sty Knockout Mice

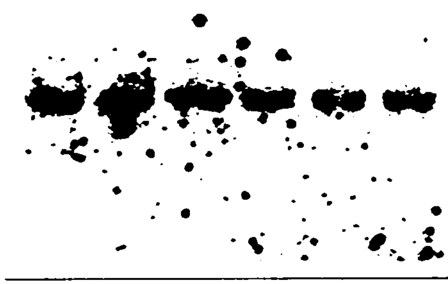
Clone 21A1 ES cells were used to generate chimeric transgenic mice. Mice which were able to transmit the Clk/ Sty disrupted allele were propagated and bred to homozygosity. No discernible phenotype was detected in mice with null alleles suggesting a possible gene redundancy. Wild type Clk/ Sty transcript is present in all tissues tested [40] but is absent in homozygous knockout mice.

Homozygous Clk/ Sty knockout mice were obtained from our colleagues to determine cellular expression of the Clk/ Sty promoter. Positive cells could be identified by X-gal staining. Histological analysis of brain (Fig 19A, B, C), kidney (Fig 19D), heart (Fig 19G), lung (Fig 19I) and testis (Fig 19E) showed that βgeo was only expressed in the testis and heart. Normally active in all tissues tested the Clk/ Sty promoter appeared to be essentially inactive in knockout mice.

Figure 18: Southern analysis of Sty Double KO ES cells show that β-geo and HygTK DNA is present in all subclones

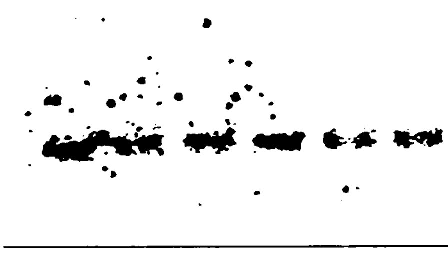
DNA was isolated from Sty double KO cells and digested with BamHI. DNA Restriction fragments were separated by electrophoresis, blotted and hybridized to lacZ (**A**) and hygromycin (**B**) probes. Appropriate sized DNA fragments were visualized using both probes.

A 1 2 3 4 5 6



3.0kb

B

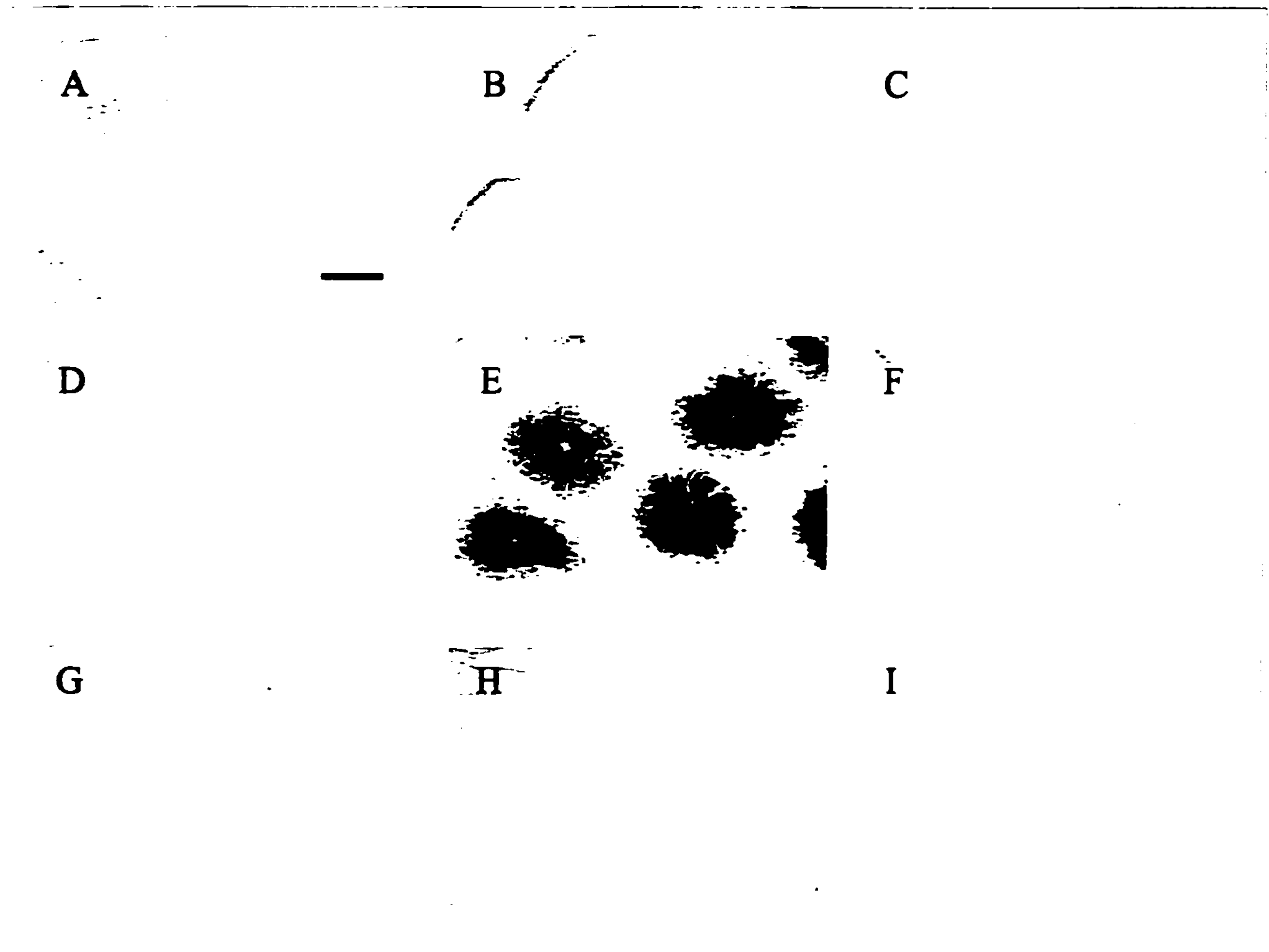


4.0kb

3.0kb

Fig 19: Expression of lacZ in various tissues from homozygous Sty double KO mice

Adult mice were anaesthesized and tissues fixed by lanas fixative perfusion through the heart. Tissues were frozen and cut into 12µm sections. Sections were X-gal stained and counterstained with nuclear fast red. Expression of lacZ was limited to the testis (**E**) and the heart (**G**). Accompanying sections from wild type animals are included; testis (**F**) and heart (**H**). LacZ staining was absent in the brain (cerebellum (**A**); cortex (**B**); dendate (**C**)), kidney (**D**) and lung (**I**). Thje bar in **A** is 10µm.

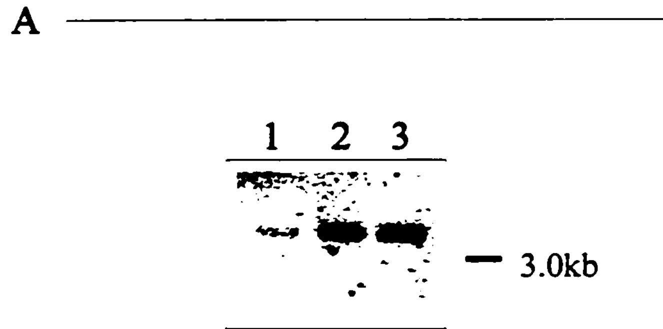

A
B
C
D
E
F
G
H
I

RNA was isolated from testis, kidney and liver of homozygous transgenic mice to test the activity of the Clk/ Sty promoter. Using a 2.3kb lacZ probe, a 5.3kb transcript was detected only in the testis (Fig 20B panel I lane 1). Transcripts were absent from kidney and liver (Fig 20B panel I lane 2,3). This suggested that the Clk/ Sty promoter was subject to gene inactivation. Insertion of a prokaryotic sequence had disrupted proper expression patterns from the Clk/ Sty promoter. LacZ sequences have been implicated in disregulation of proper expression patterns. [78] Alternatively gene rearrangement may have occurred in these transgenic mice. although expression is abundant in the testis.

A southern analysis was done to determine the presence of (geo DNA and whether rearrangements had occurred. DNA was isolated from testis. kidney, and liver, digested with BamH1. separated by electrophoresis, and analyzed using a lacZ probe. DNA fragments of expected size. 3.5kb were detected in all samples (Fig 20A). Thus the DNA appeared to be present and intact in these tissues.

Figure 20: Southern and Northern analysis of various tissues from Sty homozygous knockout mice correlates gene inactivation with loss of expression

RNA and DNA were isolated from various tissues of Sty homozygous knockout mice. These nucleic acids were separated by electrophoresis, blotted and hybridized with with a probe for E. Coli LacZ (**B,C**) and tubulin (**C**). (**A**) A figure showing the organization of the targeted region of the Sty gene with relevant restriction sites. (**B**) Southern analysis of testis (**lane 3**), kidney (**lane2**), and liver (**lane 1**) shows a 3.3kb band in all samples. (**C**) Northern analysis of testis (**lane 3**), kidney (**lane2**), and liver (**lane 1**) RNA reveals that β-geo is only expressed in the testis (**lane3**). Tubulin hybridization confirms that RNA is present in all lanes (**C lanes 1-3**)


A
1
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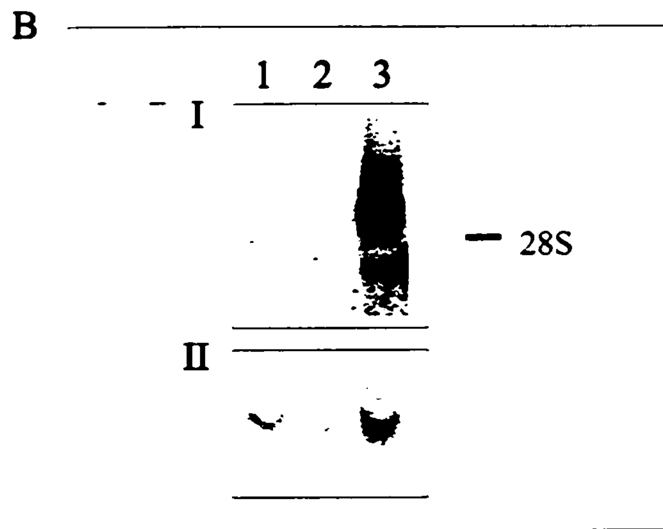

B
1
2
3
I
28S
II

Discussion and Conclusions

Stability of transgene expression is thought to be related to copy number. Single copy integrations have been suggested to result in stable and uniform expression. Similar to homologous recombination of a lacZ transgene into the active HPRT locus, homologous recombination of a promoterless βgeo sequence into the Sty locus, resulted in mosaic expression. Furthermore, we found that loss of expression was mechanistically different in ES cells and in mice derived from the same ES cells. Loss of expression in ES cells occurs as a result of gene loss and in homozygous transgenic mice it occurs as the result of gene inactivation.

X-gal staining of neomycin selected Sty KO ES cell clone 21A1 showed mosaic expression of lacZ. Examination of Sty KO ES subclones, showed two types of staining patterns. Colonies either stained mosaic or did not stain at all. Representative colonies were expanded to harvest RNA and DNA. Northern analysis confirmed loss of expression. βgeo transcripts were absent in non-expressing subclones and present in expressing clones.

DNA was isolated from the subclones to determine the nature of inactivation. We found that lacZ sequences were absent from the non-expressing clones. Thus the instability associated with homologous recombination into ES cells appeared to be gene loss. Unfortunately we cannot rule out the possibility that the original Sty 21A1 cells were not contaminated with wild type ES cells. Single cell cloning would serve to verify and directly support that gene loss is responsible for mosaicism in ES cells.

Interestingly a homozygous Sty KO clone maintained stable expression in the absence of selection. These ES cells carried an ires-βgeo cassette on one Sty allele and an ires-HygTK on the other. Single cell clones were again expanded and harvested for DNA

and RNA. LacZ transcripts and HygTK transcripts were present in all subclones and DNA sequences corresponding to the lacZ and HygTK genes appeared intact.

Gene conversion may be the mechanism of gene loss in hemizygous KO ES cells. Targeted disruption of the remaining Sty allele in homozygous Sty KO ES cells may have disrupted a sensing mechanism that corrects for mismatches during homologous pairing.

Histological and RNA analyses tells a different story in homozygous Sty KO mice. The Sty promoter is ubiquitously expressed in all tissues tested.[40] Yet, X-gal stained tissue cryosections showed that expression of lacZ directed by the Sty promoter was confined to the testis and the heart. βgeo transcripts could only be detected in the testis and not the heart. This is probably due to the limited sensitivity of northern analysis.

Again DNA was isolated to determine the mechanism of inactivation. LacZ sequences were present in all tissue samples tested. This suggested that loss of expression was due to inactivation of the Sty promoter. One possibility is that an intronic enhancer was disrupted by the βgeo cassette. If this were true ES cells would uniformly express or not express the transgene. It is expressed in a mosaic fashion.

In conclusion our findings suggest that stability of expression is more complicated that just copy number and position effects, and in fact reveals vast mechanistic differences between cell culture and transgenic animals. Gene conversion mechanisms may be responsible for loss of expression in ES cells and may require that there be one intact allele. Disruption of both Sty alleles relives mosaic expression. Loss of lacZ expression in mice appears to be due to gene inactivation. It has been previously documented that lacZ sequences may confer some cis-effects.[78] Perhaps, the activity of the Sty promoter is has been modulated by lacZ. This remains to be elucidated.

Chapter 3

Abstract

Genes transfected into embryonal carcinoma cells are both lost and inactivated at a high frequency. The inactive state in EC cells is stable and heritable. We sought to investigate whether methylation was associated with transgene inactivation in EC cells. Methylation analyses revealed that intron 1 was differentially methylated at two XhoI sites regardless of transgene expresision. Similarly, analyses of the lacZ region revealed heavy methylation of HpaII sites regardless of expression. Slight under methylation was present only in expressing cells suggesting that a small proportion of transgene copies may be unmethylated and responsible for expression within these cells.

Furthermore treatment of non-expressing cells with 5-aza-2-deoxycytidine, an analogue of 5AC, reactivated Pgk-1.2-lacZ and Pgk-Puro expression in a small percentage of treated cells. DNA isolated from 2 expanded reactivated clones correlate with demethylations within the transgene. These results support the notion that methylation is associated with transgene inactivation in EC cells.

Introduction

Transfection of exogenous genes into P19 embryonal carcinoma (EC) cells have often resulted in loss of expression.[68,94] Clones of transfected cells that express a transgene in a large proportion of cells is rather the exception. Mosaic expression of genes is a common feature of colonies of cells which were selected with a dominant marker gene. [94] Instability of transfected genes has been attributed to both gene inactivation and gene loss. [94]

Mosaic expression of transfected genes appears to be a unique feature of both embryonal carcinoma and embryonic stem cells. V79 fibroblasts co-transfected with Pgk-1,2-lacZ and Pgk-Puro form colonies under puromycin selection that either uniformly express lacZ or not at all.[94]

Methylation has been implicated in the inactivation of exogenous and endogenous genes. [108] To determine if the phenomenon observed in P19 EC cells was related to DNA methylation, stably inactivated clones of transfected P19 cells were treated with 5-Aza-Cytidine (5AC), a demethylating agent. Treatment with 5AC did not induce expression of either transfected genes. [94] The intron 1 region of the Pgk-1 gene on the inactive X-chromosome is associated with methylation. [5] However, analysis of transgenic DNA within intron 1 revealed no difference in the levels of methylation between expressing and non-expressing cells. [94] These results suggested that methylation was not the mechanism of inactivation in P19 cells.

Unconvinced by previous results we sought to duplicate experiments and perform a more detailed methylation analysis on the Pgk-1,2-lacZ transgene. We found that treatment of stably inactivated clones of transfected P19 cells with 5-Aza-2-Deoxycytidine (5A2C),

an analogue of 5AC resulted in low but reproducible reactivation of both transfected transgenes. In addition, heavy methylation was found to be associated with the Pgk-1,2-lacZ gene regardless of whether it was expressed or not. Slight under methylation was present only in expressing clones, suggesting that only a small proportion of transgene copies are active in any given cell.

Materials and Methods

Cell Culture

The cell lines , P19 4.4 and P19 4.41 were maintained as described [89]. They were cultured in minimum essential medium alpha (MEM(α) (Gibco) supplemented with 2.5% fetal bovine serum and 7.5% donor bovine serum (Cansera International Inc.). The cells were maintained in plastic tissue culture dishes, which were placed at 37°C in a 5.0% CO2 incubator. They were kept in exponential growth phase by routinely sub-culturing cells at intervals of 48 hours. Briefly, cell monolayer washed in phosphate-buffered saline (PBS) (0.8% NaCl, 0.2% KCl, 0.02% KH_2PO_4 and Na_2HPO_4), and then incubated in trypsin-EDTA (1mM EDTA and 0.025% trypsin in PBS) for 10 minutes. The detached cells were dispersed by vigorous pipetting. They were then counted and replated at a density of 250cells/mm2 into fresh medium.

Cells were exposed to various concentrations of 5-Aza-2-DeoxyCytidine (5A2C) essentially as described [81]. Cultures were grown in the presence of various concentrations of 5A2C for approximately two cell cycles; 24 hours for embryonal carcinoma , after which the drug was removed and the cells were washed and incubated for an additional 24 hours. The cultures were then harvested by trypsinization for sub-culturing or plated into 1(g/mL puromycin selection media. Puromycin resistant colonies were harvested 5 days after selection and maintained as previously described.

At various times after removal of the drug 5A2C, cells were fixed in 0.2% glutaraldehyde in PBS for 1 hour, washed and incubated overnight at 37°C in X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside).

Probes

Plasmid DNA fragments which were used as probes were isolated as follows. Briefly, plasmid DNA was digested using appropriate restriction endonucleases under the conditions recommended by manufacturer. DNA was separated by electrophoresis through 1% low melt agarose gels in 0.1X TE. The appropriate DNA fragments were isolated by through cuts into the agarose gels which were recovered by dissolving gel fragments in TE at 55°C, extracted with phenol-chloroform (1:1), and finally redissolved in TE. The LacZ probe was a 2.3kbp EcoRI-ClaI fragment from pDM2 and the α-tubulin probe was a full length mouse cDNA. Probe DNAs were 32P-dCTP labeled using the standard multiprime DNA labeling protocol (Amersham).

DNA Isolation and Electrophoresis

Genomic DNA was extracted from embryonal carcinoma (EC) cells as previously described [51]. Samples were washed in PBS and lysed in DNA lysis buffer (0.2% SDS. 200mM NaCl. 5mM EDTA, and 100mM Tris HCl (pH 8.5) containing 100(g/mL proteinase K). After incubation at 37oC for 2 hours with gentle agitation, one volume of isopropanol was added to recover DNA from the lysate. The DNA was dissolved in TE (10mM Tris HCl (pH 8) and 1mM EDTA).

Purified genomic DNA was digested by restriction endonucleases at a concentration of 10U/ μg. DNA fragments were separated by electrophoresis through 1.0% agarose gels in 0.1X TE. Separated DNAs were transferred to Hybond-N membranes (Amersham) and treated with ultraviolet light at 110mJ using a GS Gene linker UV chamber (Biorad).

Northern Blot

RNA was isolated using TRIzol (Gibco BRL). Briefly, monolayer cells were lysed by addition of TRIzol. Following incubation at room temperature for 5 minutes 0.2mL chloroform/ mL TRIzol was added to the sample and incubated for an additional 2 minutes. Samples were centrifuged at 12,000g for 15 minutes at 4°C and aqueous phase mixed with 0.5mL isopropanol/ mL TRIzol. Following a 10 minute incubation at room temperature, samples were centrifuged at 12,000g for 10 minutes at 4°C. After washing with 70% ethanol, the RNA was dissolved into Rnase-free water.

RNA was separated by electrophoresis through 1% agarose gels in 20mM 3-[N-morpholino]propane-sulfonic acid, 1mM EDTA, 5mM sodium acetate at pH 7, and 10% formaldehyde. Separated RNAs were transferred to Hybond-N membranes (Amersham) and treated with ultraviolet light at 110mJ using a GS Gene linker UV chamber (Biorad).

Probe Hybridization

Prehybridization was carried out in 50% deionized formamide, 5 x SSPE, 2.5 x Denhardt's solution, 0.1% SDS, 0.2mg/mL denatured salmon testis DNA (1 x SSPE is 0.15M NaCl, 10mM $Na_2H_2PO_4$, and 1mM EDTA). Membranes were hybridized for 15-24 hours at 42oC in prehybridization solution containing 32P-dCTP labeled probes. Following incubation, membranes were washed once in 2 x SSC and 0.1% SDS at 42°C for 15 minutes, followed by several changes of 0.2 x SSC and 0.1% SDS at 65°C for 45 minutes. Radioactivity was visualized and analyzed using Molecular Dynamics Phosphorimager SI.

Results

Treatment of inactive transfected genes with 5A2C result in reactivation

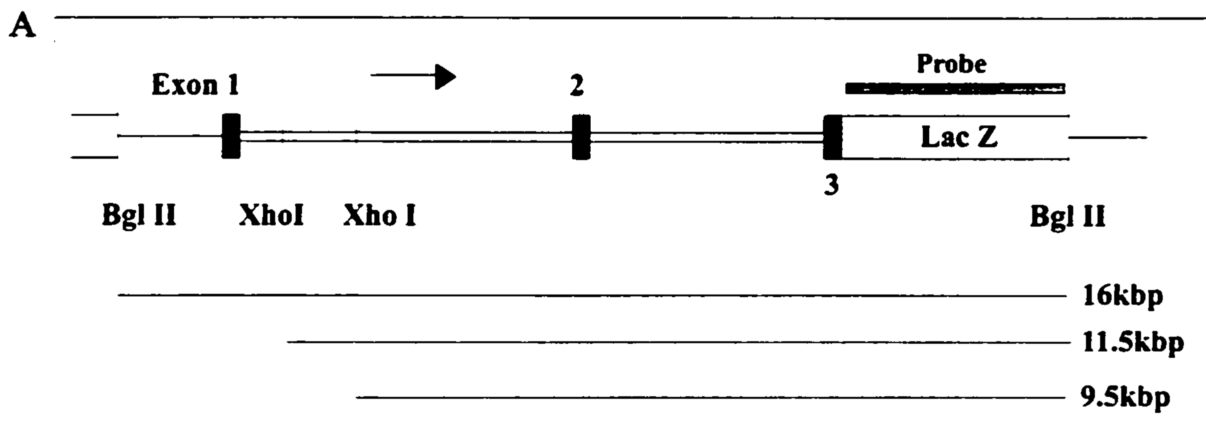
P19 cells were previously co-transfected with Pgk-1,2-lacZ encoding a (-galactosidase fusion protein, and Pgk-Puro which confers selective resistance to puromycin. [94] The Pgk-1 promoter drives expression of both genes which also carry polyA and termination signals. The Pgk-1 promoter has previously been shown to be very active in EC cells. [68]

P19 clones with inactive transgenes were treated with 5AC with no discernible effects. [94] Treatment of the same clones with 0.5(m and 3.0(m 5A2C, an analogue of 5AC, for 24hr, resulted in 2.6±0.7% (Std. Dev) and 6.4±1.7% (Std. Dev) reactivation of Pgk-1,2-lacZ, respectively (Fig 21A) . Reactivation of Pgk-1,2-lacZ is heritable since an increasing number of blue staining cells were present as groups at 48hr as compared to 24hr after treatment (Fig 21B).Untreated P19 clones spontaneously reactivate the lacZ gene in < 1 in 10^{-6} cells.

Selection of 5A2C treated P19 clones in 2µm puromycin gave rise to 400 colonies out of $5*10^5$ plated cells. A representative portion of colonies were selected for subculture. The rest of the colonies were fixed and stained with X-gal. Stained colonies were either uniformly blue, mosaic or white (Fig 21C). The presence of a large number of mosaic colonies suggested that reactivated transgenes are also prone to instability, the nature of which remains to be elucidated. Selected colonies were expanded for DNA isolation, as well as X-gal staining. β-galactosidase negative cells were present in all expanded colonies. We don't know however, that selected colonies were originally contaminated with white cells.

Figure 21: The intronic regions of Pgk-1,2-lacZ are differentially methylated in expressing and non-expressing cells.

(**A**) A diagram of the Pgk-1,2-lacZ transgene shows restriction sistes for BglII and XhoI within the intronic region, along with a reconstruction of various detected fragments. (**B**) DNA was isolated from P19 control cells (**lane 1**), lacZ expressing P19 cells (**lane 2**), an inactivated clone P19 441 (**lane 3**), and 5A2C induced P19 441 clone D1 (**lane 4**) and D2 (**lanes 5**) . 10ug of DNA was digested with BglII and methylation sensitive XhoI restriction enzymes, separated on a 1.0% agarose gel, transfered to nylon and probed with a 2.3kb lacZ fragment. Intronic methylation profiles show little difference between expressing P19 44 cells (**B lane 2**), expressing 5A2C induced D1 clone (**B lane 4**) and non expressing 919 441 cells (**B lane 3**). 5A2C treated P19 441 clone D1 showed complete demethylation within the intronic region (**B lane 5**).


A
Probe
Exon 1
2
Lac Z
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XhoI
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Bgl II
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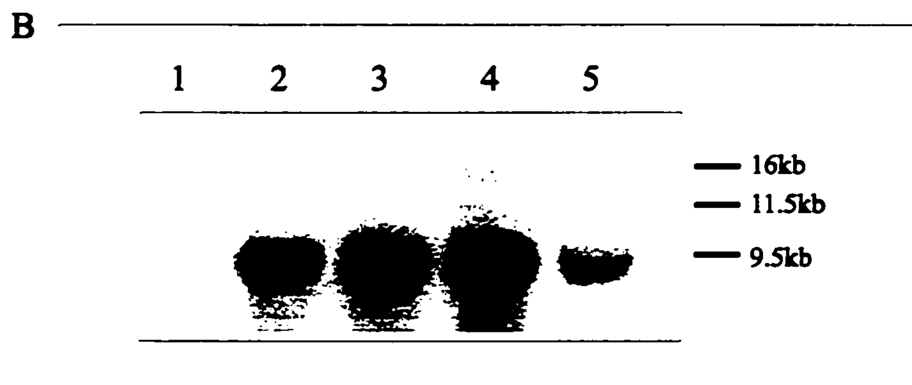
B
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2
3
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16kb
11.5kb
9.5kb

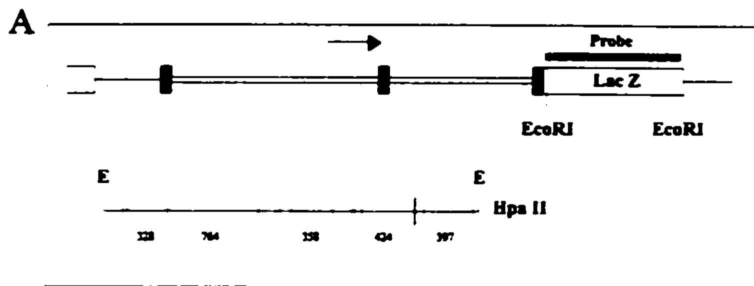
DNA Methylation is Associated with The LacZ Gene in P19 Cells

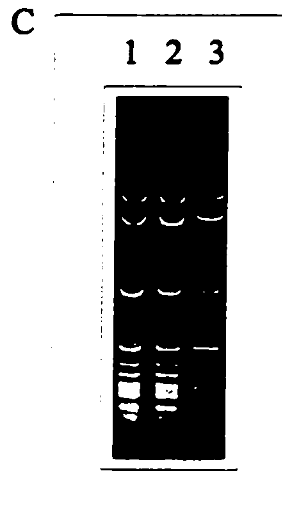
An examination of the methylation within the Pgk-1,2-LacZ transgene was previously done. The intron 1 region of the transgene was differentially methylated but levels of methylation were indifferent between expressing and non-expressing cells. [94] The Pgk-1 gene is located on the X chromosome and is methylated at two XhoI sites in intron 1 on the inactive X chromosome. [5] We reinvestigated methylation of these two XhoI sites in expressing, non expressing and two 5A2C reactivated clones. Restriction sites for BglII are located once in Pgk-1,2-lacZ and Pgk-Puro plasmids and yields a 16kb fragment upon digestion and hybridization to a lacZ probe. Subsequent digestion with methylation sensitive XhoI depending on unmethylated recognition sites yields three fragments of sizes 9.5, 11, and 16kb (Fig 22A). The three bands were present in all non expressing and expressing clones except 5A2C reactivated clone D8 (Fig 22B). Digestion of DNA from clone D8 yielded only a 9.5kb fragment suggesting that no methylation was associated with intron 1 of the transgene, and may been induced by drug treatment (Fig 22B lane 5). Clone D1 is also a reactivated 5A2C clone but no differences in methylation were observed within the intron 1 region (Fig 22B lane 4).

We went on to investigate methylation within the lacZ region of the Pgk-1,2-lacZ region. Prokaryotic sequences have been known to be associated with gene inactivation and methylation. [8,78,97] Inactivation of a keratin 18/ lacZ fusion was correlated with methylation within the lacZ gene. [106] EcoRI restriction sites flank the lacZ gene and upon digestion yields a 3.0kb fragment. HpaII is a methylation sensitive enzyme and Msp I, its isoschizomer is methylation insensitive. There are multiple HpaII restriction sites within lacZ.

Figure 22: HpaII sites in the lacZ gene are heavily methylated regardless of expression.

(**A**) DNA was isolated from P19 control cells (**lanes 1,2,3**), lacZ expressing P19 clone (P19 44) (**lanes 7,8,9**), an inactivated clone P19 441 (**lanes 4,5,6**), and 5A2C induced P19 441 clones D1 (**lanes 10,11,12**) and D2 (**lanes 13,14,15**) . 10ug of DNA was digested with EcoRI (**lanes 1,4,7,10,13**), EcoRI with MspI (**lanes 2,5,8,11,14**) and EcoRI with HpaII (**lanes 3,6,9,12,15**). The Pgk-1,2-lacZ gene was heavily methylated within the lacZ region (**lanes 6,9,12,15**). Slight demethylation is present within expressing cells P19 44 (**lane 9**) and demethylated clone D1 (**lane 12**) indicated by restriction fragments <3.0kb. The lacZ probe does not hybridize to DNA within P19 negative controls (**lanes 1,2,3**). Shown in (**B**) is an accompanying ethidium bromide stained gel visualized under UV illumination. (**C**) An ethidium bromide stained gel shows that digestion of DNA is complete with EcoRI and HpaII under selected conditions. Plasmid alone (**lane 3**), plasmid seeded in genomic DNA (**lane 2**) digested with EcoRI and MspI, and plasmid seeded in genomic DNA (**lane 1**) digested with EcoRI and HpaII.


A
Probe
Lac Z
EcoRI
EcoRI
E
E
Hpa II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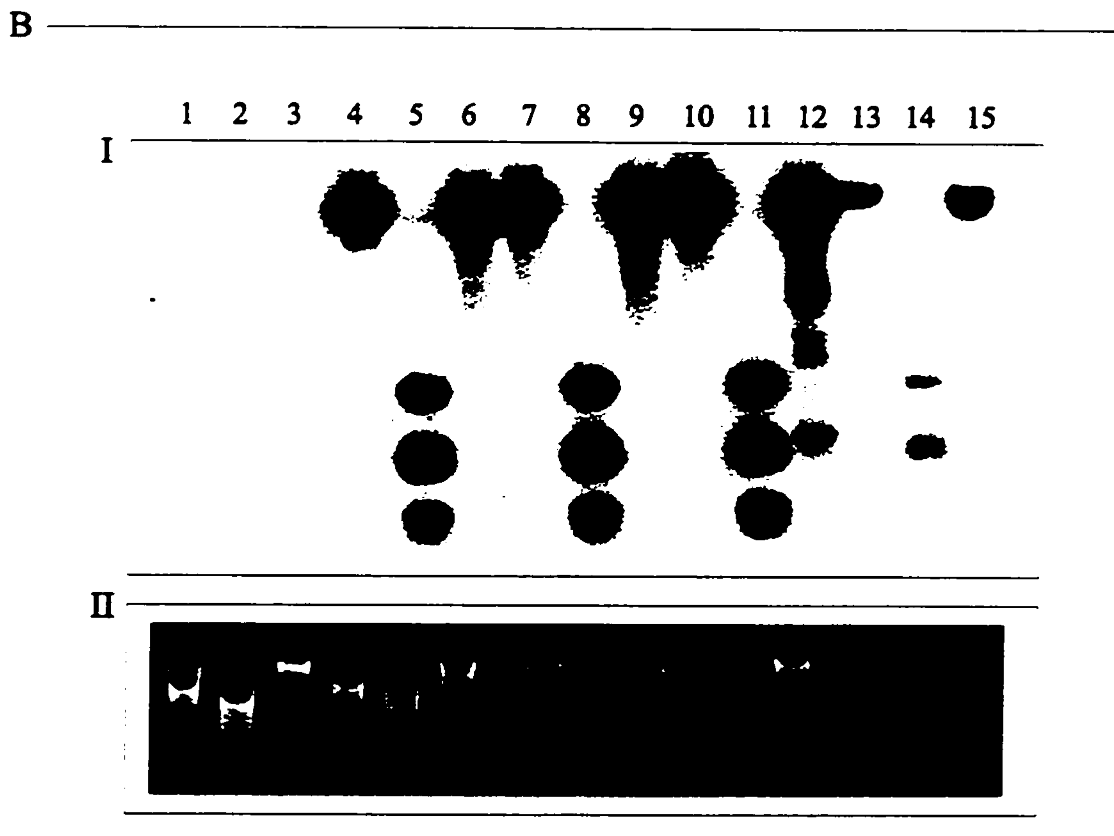
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II

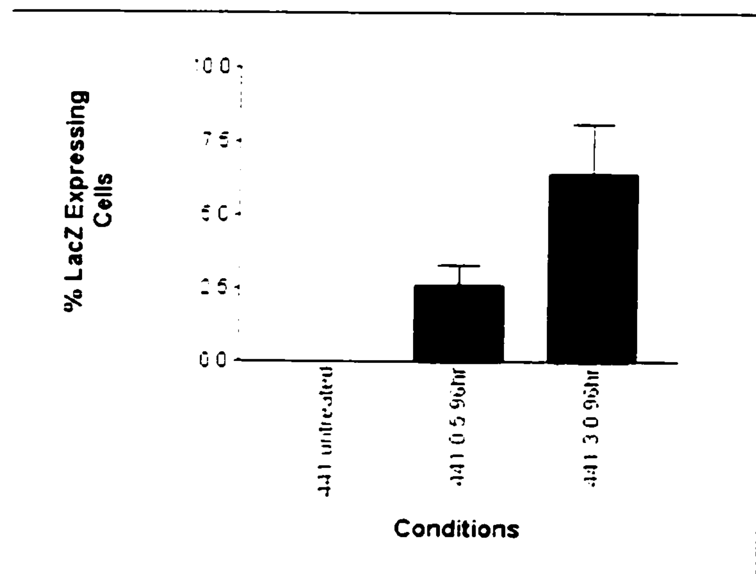
Subsequent digestion of EcoRI digested Pgk-1,2-lacZ plasmid with MspI yields many fragments which resolve into three major bands at 700, 350 and 100bp. HpaII digests yield the same MspI restriction patterns if no methylation is present. (Fig 23A)

All DNAs digested with EcoRI and HpaII yielded a predominant 3.0kb band indicating that the lacZ gene is heavily methylated (Fig 23B). Clone D1 yielded a significant proportion of bands which deviated from MspI digestion patterns which suggested that the lacZ gene was differentially demethylated by 5A2C treatment (Fig 23B lane 12). P19 44 (transgene expressing) cells also have two additional HpaII fragments at 700bp and 350bp, which may indicate that a small proportion of transgenes are under methylated (Fig 23B lane 9). No additional HpaII fragments were resolved in clone D8, which was found to be demethylated in the intron 1 region (Fig 22B lane 15). Additionally copy number, within this clone may have been altered upon 5A2C treatment, if loading was equivalent (Fig 23B panel II). To assess that digestion went to completion plasmid DNA was digested in the presence and absence of genomic DNA (Fig 23C).

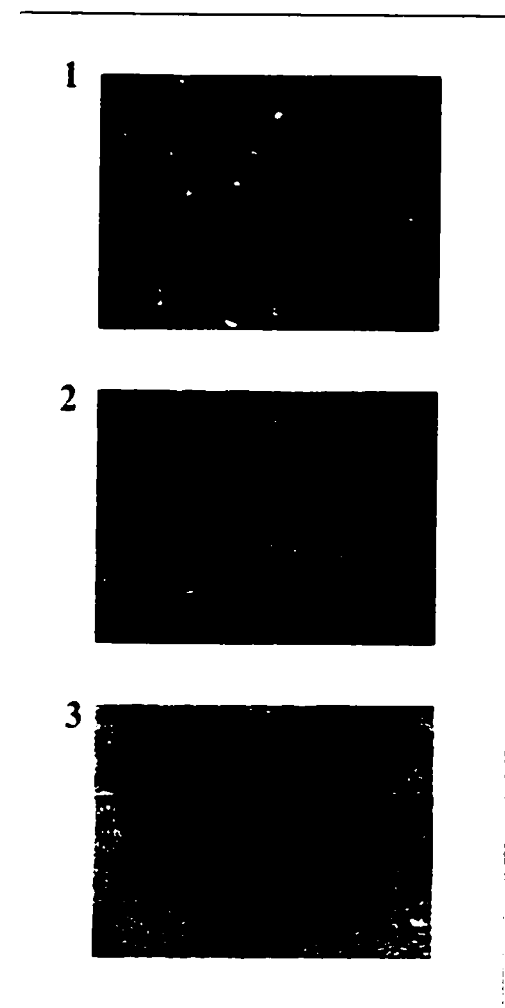
Figure 23: Dose responsive reactivation of transgene expression in non-expressing cells treated with 5-aza-2-deoxycytidine.

P19 441 cells were treated with 0.5μm and 3.0μm 5A2C for 24hr. Subsequent fixation with 0.02% glutaraldehyde and staining with X-gal resulted in Pgk-1.2-lacZ expression in 2.6±0.7% and 6.4±1.7% of treated cells, respectively (**A and B panel 2,3**). Reactivation of the transgene is appears to be heritable as indicated by clusters of blue cells or Pgk-1.2-lacZ expressing cells (**B panel 3**) after 48hr as opposed to 24hr in culture (**B panel 2**). Selection of 5A2C treated cells in 2% puromycin, induced colony formation in approximately 400 of $5*10^5$ cells and subsequent staining with X-gal revealed that colonies were all blue (**C lane 3**), mosaic (**C lane 2**) or white (**C lane 1**). Untreated P19 441 cells express Pgk-1.2-lacZ in $<1*10^{-6}$ cells and fail to produce any colonies when selected in puromycin. (n=3)

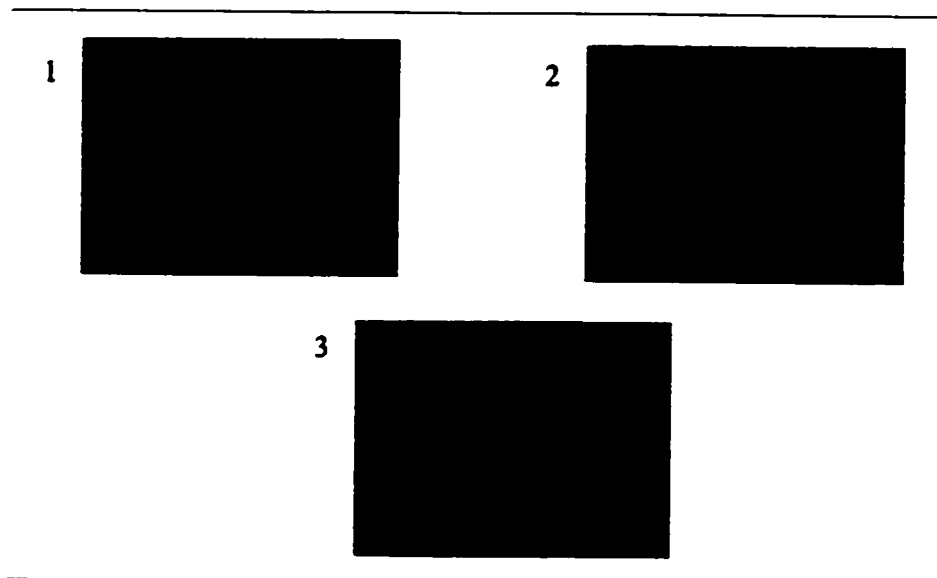
A



C



B



Discussion and Conclusions

Not dissuaded by previous results, we did a more detailed examination of methylation within the Pgk-1,2-lacZ transgene and repeated demethylation experiments using 5A2C, a 5AC analogue. Re-examination of methylation within the intron 1 segment of Pgk-1,2-lacZ using methylation sensitive XhoI restriction enzymes showed that it was partially methylated but no difference in methylation was found between expressing and non-expressing clones. We then analyzed methylation within the lacZ region, using MspI and methylation sensitive HpaII. Methylation has been found to be correlated with inactivation of a K18-lacZ transgene in mice [106] and present within the lacZ region of the same transgene construct in our transgenic mice. We found that the lacZ region was heavily methylated with slight under methylation present only in expressing clones.

Transfected genes integrate in multicopy tandem repeats. It is unknown how many transgene copies are active in any one cell. All transgene copies are presumed to be silent in non-expressing cells. Our results suggest that a small proportion transgene copies are active in expressing clones. In yeast and *Drosophila*, the spreading of inactivation, from a region of heterochromatin is dependent on concentrations of repressive and activating factors.[4] Increasing transcriptional activators alleviates repressive effects on telomere-proximal genes, in yeast. [4] Thus, derepression of genes seemingly occurs at the boundary of repressive complexes. A similar situation may exist in cultured cells and may suggest that active Pgk-1,2-lacZ genes within transgene concatemers are located at the boundaries of inactive transgene clusters.

The use of demethylating agents has widely been used to correlate inactivation and methylation. We repeated experiments using a 5AC analogue, 5A2C. Treatment of inactive

P19 clones with 5A2C resulted in a dose dependent reactivation of Pgk-1,2-lacZ as well as reactivation of Pgk-Puro. Reactivation of Pgk-1,2-lacZ was heritable and occurred in 6.4±1.7% (Std. Dev) of treated cells with a dosage of 3.0μm 5A2C. Untreated cells spontaneously reactivate the transgene in $< 1*10^{-6}$ cells. Selection in puromycin yielded 400 colonies out of $5*10^5$ plated cells. Although, both transgenes were reactivated upon 5A2C treatment, the level of reactivation was low. It is possible that demethylating agents are inefficient in EC cells leading to a low amount of reactivation. This suggestion is consistent with the failure of 5AC to reactivate transgene expression in P19 EC cells.

Puromycin resistant clones were fixed and stained with X-gal. Colonies of cells either stained uniformly blue, did not stain at all, or were mosaic. Majority of resistant colonies were mosaic suggesting that reactivated transgenes were also prone to instability.

If 5A2C was responsible for reactivation of Pgk-1,2-lacZ, there should be evidence supporting demethylation of the transgene. We selected two puromycin resistant 5A2C reactivated clones, and subject them to methylation analyses within the intron 1 and lacZ regions. In clone D1, no difference in intron 1 methylation was detected, however, demethylation was evident within the lacZ region. Clone D8, showed the complete opposite; complete demethylation within the intron 1 region but no detectable demethylation within the lacZ region.

Demethylation of the Pgk-1,2-lacZ transgene was detected within the two 5A2C treated clones but within two different regions in each of the clones. These results make it difficult to say for certain that 5A2C reactivation is directly related to demethylation of our transgene. One possibility is that the overall decrease in methylation density over the entire

transgene is responsible for its reactivation. There is evidence that methylation density can control the extent of gene repression. [6]

Despite heavy methylation within the lacZ region, slight under methylation is detected only in wild-type expressing clones suggesting that a small proportion of transgenes are active in a given cell. Furthermore, both Pgk-1,2-lacZ and Pgk-Puro are reactivated upon treatment with demethylating agent 5A2C. Two 5A2C reactivated clones show demethylation within the Pgk-1,2-lacZ transgene. Methylation may thus be the mechanism of inactivation of transgenes within EC cells.

General Discussion and Conclusions

We have shown that a single line of Pgk-1,2-lacZ transgenic mice is prone to gametic imprinting. Maternal transmission of the transgene results in irreversible inactivation of the transgene. The irreversibility of the transgenic imprint contrasts natural genomic imprinting which is reversible. There exists the possibility that genes may irreversibly inactivate, but a more plausible explanation would be that the irreversibility is an artifact. Nevertheless, the irreversibility of the transgenic imprint may allow us to study the nature of the germline, which restores genomic totipotency by erasing all epigenetic modifications.

The lacZ region was found to be heavily methylated at HpaII/ MspI sites regardless of tissue expression. Slight undermethylation is only present in expressing tissues, suggesting that a fraction of transgenes in a transgene array are active and may be responsible for expression. The Pgk-1,2-lacZ transgene exists in a head to tail array of 6 copies (results not shown). We are uncertain whether active transgenes exist at the border or within the interior of the array. Experiments in yeast suggest that inactivation can spread into a transgene from a heterochromatic region and can be alleviated by insertion of an upstream enhancer.[4] It has been proposed that there exists a competition between activating and repressive factors that occurs at the boundary of repressive complexes.[4] Experiments are underway to determine where active transgenes are located in the transgene array.

The use of demethylating agent 5-aza-2-deoxycytidine on MEFs carrying maternally inherited transgenes resulted in reactivation in a small percentage of cells. Together with methylation data, these results suggest a correlation between methylation and inactivation.

Similar but unrelated experiments in P19 EC cells also reveal that the Pgk-1,2-lacZ transgene is heavily methylated within the lacZ region regardless of expression. Slight undermethylation is only present within expressing clones, again suggesting that a fraction of transgene copies are active within a transgene array. Treatment of inactivated P19 clone 441 containing Pgk-1,2-lacZ and Pgk-Puro, with 5-aza-2-deoxycytidine, resulted in reactivation of both transgenes. To correlate reactivation with demethylation, we selected two reactivated clones and subjected them to methylation assays in the lacZ region and in intron 1. Clone D8 showed demethylation within the intron 1 region of Pgk-1,2-lacZ, whereas clone D1 showed demethylation within the lacZ region. Methylation density has been shown to affect expression.[6] Perhaps a decrease in methylation density within the Pgk-1,2-lacZ transgene within the two reactivated clones alleviated repression.

Initially, no differences in expression patterns were noted in the original strain despite, male or female transmission.[69] Imprinting effects occurred after the single line was bred to homozygosity. Additional Pgk-1,2-lacZ transgenic animals were generated with the hope of duplicating imprinting effects in the original strain. Over 30 founders were generated. 19 founder animals were sacrificed for analysis of DNA methylation patterns and histological analyses. Methylation was found within the lacZ region. This suggested that methylation was established during development. Again, only slight demethylation was present within highly expressing tissues supporting the notion that only a fraction of transgenes are active within a transgene array. Histological analysis showed that there was great variability in the level and pattern of transgene expression between the different founders, which lead us to believe that the Pgk-1,2-lacZ transgene is prone to position

effects. Mating experiments are still underway to determine if imprinting effects can be duplicated.

Unusual transgene effects in mice have been attributed to copy number, position of integration, strain specific modifiers, and cis-effects. Evidence from *Drosophila* suggests that copy number is directly responsible for the transgene inactivation.[23] However, homologous recombination of a knockout vector into the HPRT gene, in mice, resulting in a single copy integration, did not insulate the reporter gene from inactivation.[96] Dr. John Bell's lab had created a Clk/ Sty knockout in J1 Mouse ES cells and a transgenic knockout mouse. Expression of the reporter gene driven by the endogenous Clk/ Sty promoter was heterogeneous in ES cells and almost absent in knockout mice. The advantage of using the Clk/ Sty knockouts was that expression is being driven from an unaltered endogenous promoter driving a promoter-less construct. The HPRT knockout mice contained a reporter driven by an exogenous promoter which may have been modulated by neighbouring regulatory elements.

We found that heterogeneity of reporter expression in hemizygous Clk/ Sty knockout ES cells was due to gene loss. In Clk/ Sty double knockout mice, heterogeneous reporter expression was due to transgene inactivation. There exists the possibility that the reporter construct contains regulatory elements which affect the endogenous Clk/ Sty promoter. If this were true we would expect homogeneous expression or silencing in the ES cell knockouts.

These results suggests that the regulation of transgene expression is more complicated than originally thought. Controlling copy number and position of integration was not able to alleviate unusual transgene effects.

The use of transgenic technologies has allowed us to understand the regulation and biological significance of many genes. We are expecting to use gene therapy technology to cure diseases and genetic disorders. Unfortunately, the introduction of exogenous genes into the genome is still not well understood. Many unusual and unexpected transgenic effects have been documented. It is thus important to study how transgenes are affected by or are affecting, the environment in which they integrate. Transgenic mice first anticipated the existence of naturally imprinted genes. The unusual Pgk-1,2-lacZ transgenic line may provide insights into both, the nature of transgene inactivation and the nature of the germline.

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   S.S.K. Lau and M.W. McBurney. Manuscript in preparation

Abstract

1. S.S.K. Lau and M.W. McBurney. The Study of Gene Inactivation in Pgk-1,2-LacZ Transgenic Mice and P19 Embryonal Carcinoma Cells. (1997) 17th Annual Great Lakes Mammalian Development Meeting, April 11-13, Mount Sinai Hospital, Toronto, Ontario.

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IMAGE EVALUATION
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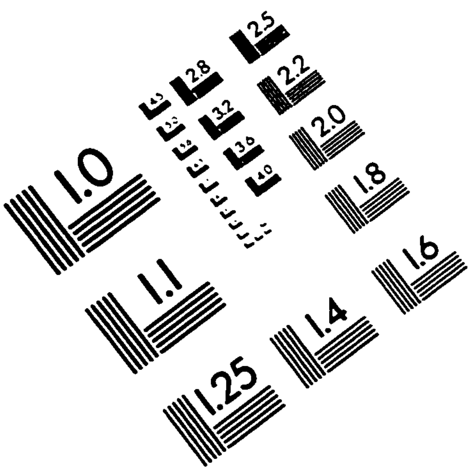

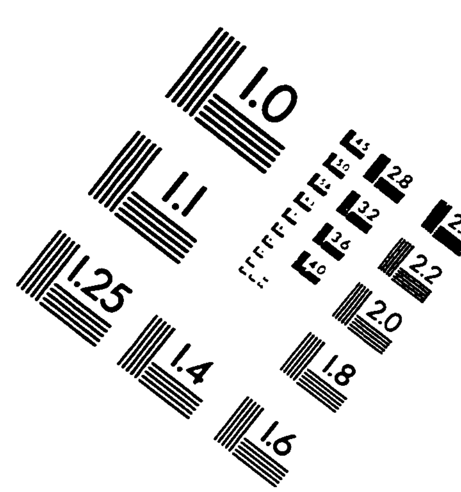

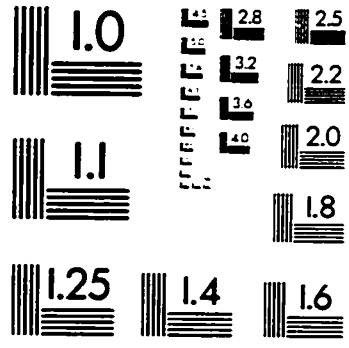

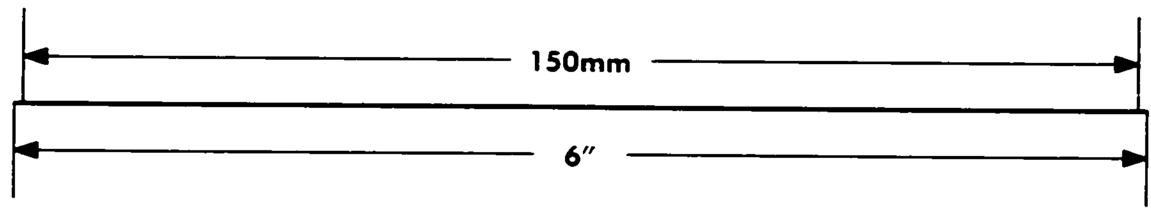

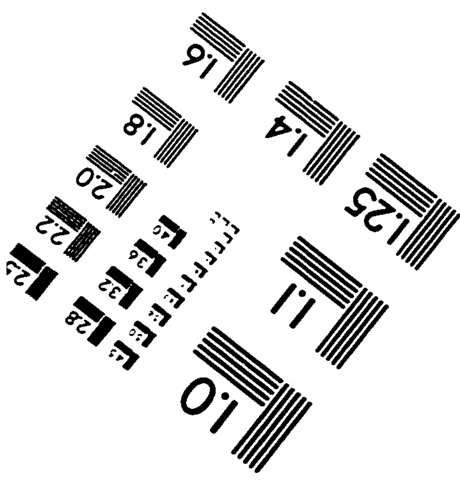

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